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Influence of Added Dead Space on Pulmonary Ventilation.

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The problem of the extent of non-uniform gas distribution in the human lungs continues to be the subject of many studies, both from the experimental and clinical point of view. In earlier experiments (BOUHUYS, HAGSTAM and LUNDIN 1956) we studied the influence of moderate degrees of exercise upon the degree of non-uniform ventilation. We found no significant differences in this series, where the tidal volume ranged from about 0.5 to 2.5 litres. We used two indices of ventilatory efficiency, *i. e.* the percent nitrogen clearance delay (FOWLER, CORNISH and KETY 1952) and the lung clearance index (BECKLAKE 1952). The first index is designed primarily to assess the degree of non-uniformity, whereas the second expresses the ventilatory efficiency as a whole in terms of ventilatory volume used to wash out one litre of the lung volume. Neither of these indices changed significantly in the range of tidal volumes mentioned. These results are in accordance with those of other workers (ROBERTSON, SIRI and JONES 1950, LUSK, HICKAM, BONDURANT and FRAYSER 1956), but RAUWERDA (1946) concluded that ventilation became more uniform during hyperventilation in his experiments. However, most of RAUWERDA's experiments were performed with two subjects, and in those experiments where the improved distribution could be expressed quantitatively, the difference appears to be rather small (RAUWERDA loc. cit., p. 78). Our results could be explained by assuming an increase in dead space proportional to

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the increase in tidal volume. Theoretically, an unchanged over-all efficiency could also be explained by the assumption of a constant dead space with increasing tidal volume, with a larger degree of non-uniformity, or by the occurrence of changes in both dead space and degree of non-uniformity which cancelled out each other with respect to over all ventilatory efficiency.

To pursue the problem further, we have studied hyperventilation induced by adding an extra dead space to breathing, and we have tried to make a distinction between the changes in ventilatory distribution and possible changes in the subject's dead space during these experiments. The problem of the dead space has been reviewed recently by ROSSIER and BÜHLMANN (1955). The behavior of calculated dead space values with changes in tidal volume depends much on the method used. If the dead space is measured with a gas with minimal alveolo-capillary exchange, such as nitrogen, helium or argon, the dead space volume calculated corresponds more or less to the volume of the conducting airways, the anatomical dead space, and shows an increase with increasing lung volume (FOWLER 1948) but only small variations with changes in tidal volumes. With such methods a significant change in dead space volumes is evident only at very small tidal volumes (LOEWY 1894, BRISCOE, FORSTER and COMROE 1954). If, on the other hand, the respiratory gases, O_2 oxygen or carbon dioxide, are used for determining the dead space, one measures not only the volume of inspiratory air which does not mix with alveolar air, but also lung spaces with reduced perfusion in proportion to ventilation and the rate of gas exchange between alveoli and blood will influence the dead space values. The calculated dead space will show, on the average, a linear increase with tidal volume (ENGHOFF 1932, 1938, ROSSIER and BÜHLMANN loc. cit.). Such a method measures the physiological dead space. In the terminology of PAPPENHEIMER, FISHMAN and BORRERO (1952, also FOLKOW and PAPPENHEIMER 1955) the anatomical dead space is called the 'series' dead space and the well-ventilated, poorly perfused lung spaces the 'parallel' dead space. These investigators found that the series dead space had a constant value for all tidal volumes in the range tested. In normal subjects a parallel dead space was present only in special circumstances (pressure breathing). WILLIAMS and RAYFORD (1956) pointed out, however, that one of PAPPENHEIMER's assumptions (*i. e.* that the extrapolated alveolar carbon dioxide tension equals the arterial pCO_2) was not correct.

For this reason, PAPPENHEIMER's results could also be explained by assuming the dead space to equal a constant plus a value varying in size with the tidal volume.

In the following we shall discuss our own measurements of the dead space with a method involving a mixing criterion. Measurement of the dead space volume was, however, not the primary aim in this investigation. Dead space calculations were included only in order to permit evaluation of the changes in ventilatory distribution and over-all efficiency in our experiments.

Various investigators have tried to check measurements of the respiratory dead space by measuring additional dead spaces. One source of errors in this respect is that the values only fit in with the anatomical dimensions of such an additional dead space if this is ventilated completely during the experiment. In the cylindrical tubes most commonly used as extra dead spaces, a certain degree of axial flow at lower tidal volumes must be expected. In our experiments, errors due to axial flow in the extra dead space were negligible (see Methods). The effects of an extra dead space upon the tidal volume, respiratory rate and blood gases has been studied by a number of authors (LILJESTRAND 1918, SEGALL 1934, STANNARD and RUSS 1948), but in many studies the incomplete wash-out of the extra dead space during breathing was not taken into account. We do not know of any publications on the influence of extra dead space upon the distribution of ventilation.

Methods.

In general the methods used were the same as those described in our previous paper (BOUHUYS et al. 1956). Experiments were performed with the subject in a semireclining position. An open-circuit nitrogen wash-out method with continuous recording of end-tidal nitrogen concentrations with an A—C nitrogen meter was used. The expired air was collected in a 100-litre TISSOT spirometer. The nitrogen wash-out was considered to be complete when the end-tidal nitrogen concentration had decreased from the initial 80 % to 2 %. The duration of the wash-out period was determined (wash-out time); tidal volume, respiratory rate and minute volume were measured from the spirographic record. The functional residual volume, the lung clearance index (= total ventilation divided by functional residual volume, both in litre BTPS), and the fractions of the lung volume were determined as described in the earlier paper (loc. cit.). Every experimental series consisted of one wash-out period at rest in a relatively basal state, and two wash-out periods with extra dead spaces of 260 and 480 ml, respectively. These extra dead spaces consisted of pieces of rubber

tubing interposed between the breathing valve and the mouthpiece. Their volumes were measured by filling with water. One needle-valve sampled expiratory air at the distal end of the extra dead space, just beyond the expiratory valve; the experimental data were derived from these records. Another needle-valve was incorporated in the extra dead space close to the mouthpiece. In a number of experiments this needle-valve was connected to a second nitrogen meter; these records showed that at the end of each inspiration of oxygen the nitrogen concentration near the mouthpiece decreased to near zero, which indicates that in the subjects studied the extra dead space was always completely washed out with oxygen during each inspiration. Therefore we consider the 'anatomical' volume of the extra dead space to equal the 'physiological' volume of the same. Between wash-out periods we allowed a 15 to 20 minutes rest for re-nitrogenation of lungs, blood and tissues with a rapid nitrogen elimination rate. In the runs with extra dead space, the subject breathed air through the dead space for 6—7 minutes to obtain a steady state before the start of the wash-out period. For temperature corrections we measured the temperature in the middle of the extra dead space and in the TISSOT spirometer.

From the calculation in the previous paper it may be recalled that each fraction of the lung volume is characterized by its fractional volume and its dilution factor w, which indicates the ventilation rate of the fraction. From these properties one can define the average number of breaths during which a nitrogen molecule remains in the lungs before it is washed out (actual number of breaths). One can also calculate an ideal dilution factor W: the dilution factor of a system with the same functional residual volume, tidal volume and dead space as the system under study, but with a uniform ventilation rate. From the ideal W we calculated the average ideal number of breaths, *i. e.* the number of breaths a nitrogen molecule would remain in a system of the same dimensions but ventilated uniformly. These calculations were developed by FOWLER et al. (1952). The alveolar dilution factor equals: $\frac{V_L}{V_L + (V_T - V_D)}$,

where

V_L = functional residual volume,

V_T = tidal volume,

V_D = respiratory dead space volume.

Therefore, if we determine the ideal dilution factor W, the functional residual volume and the tidal volume, V_D can be calculated. This is the dead space of a theoretical, uniformly ventilated system, but owing to the definition of the ideal dilution factor W, the same dead space must exist in the experimental, non-uniformly ventilated system.

Some of the results have been subjected to statistical analysis. The regression of dead space on tidal volume has been calculated with the method of least squares. The equations used, and also those for the calculation of correlation coefficients, are those given by MONRONEY (1953). The significance of the differences between the average delay percentages and the average dead space volumes has been tested with the 'sign test' of DIXON and MOOD (1946).

Results.

The results of 15 experiments with 5 normal subjects are given in Table I. Table II shows the average values. There is no consistent change in the respiratory rate when an extra dead space is added, nor does the functional residual volume show important alterations. On the whole there is a small increase with the larger extra dead space. The increase in tidal volume is on the average as large as the volume of the extra dead space, a fact noted previously by LILJESTRAND (1918). The average actual number of breaths a nitrogen molecule remains in the lung spaces increases with the addition of a dead space. This points to a slowing of the course of the wash-out process. The average number of breaths calculated for the ideal case, however, also increases markedly, and the

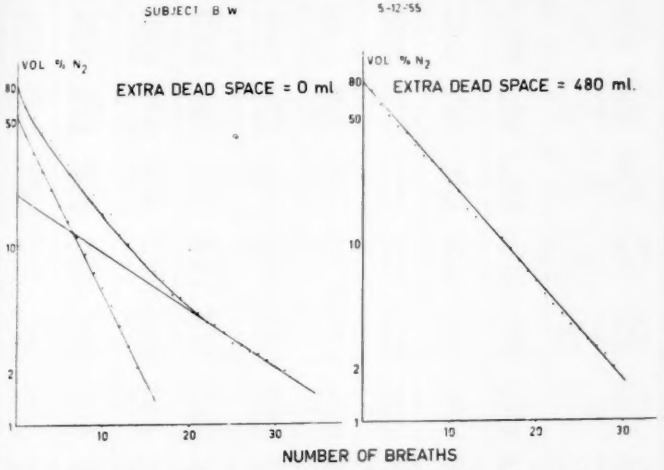

Fig. 1. Semilog plots without extra dead space (left) and with extra dead space of 480 ml (right).

difference between the actual and the ideal number of breaths (the delay percentage) decreases significantly. The delay percentage is a measure of the degree of non-uniform ventilation; thus these findings suggest a slowing of the wash-out process but at the same time a more uniform ventilation. The slowing of the course of the nitrogen wash-out is also illustrated by the lung clearance index. We have calculated this index in two ways: firstly for the total

Table

Results of 15 experiments

Subject	D. S. added ml	Total no. of breaths	Wash-out time min.	Resp. freq. /min.	Total vent. L	Tidal air ml	Vol. O_2 wash-out lungs L	Tidal vol. wash-out lungs ml	FRA + extr. D. S. L
L. Å.	0	36	2.6	13.7	23.3	647	23.3	647	2.6
27/10—55 ..	260	52	3.3	15.6	39.8	765	26.3	506	2.8
	480	64	3.6	17.9	59.0	922	28.6	447	3.1
L. Å.	0	55	3.3	16.8	23.1	420	23.1	420	2.7
1/12—55 ...	260	54	3.3	16.4	28.3	709	23.6	438	3.3
	480	69	4.2	16.4	61.8	896	28.8	479	4.0
B. W.	0	29	3.1	9.4	23.2	800	23.2	800	4.4
3/12—55 ...	260	33	3.6	9.2	31.3	948	22.6	687	3.9
	480	41	4.3	9.5	45.9	1,120	26.2	641	4.5
B. W.	0	33	3.4	9.7	22.8	691	22.8	691	3.4
5/12—55 ...	260	34	3.8	8.8	32.1	944	23.2	683	4.0
	480	32	3.7	8.6	40.9	1,278	25.6	799	4.3
T. R.	0	39	2.9	13.4	22.3	572	22.3	572	3.0
7/2—56	260	32	3.1	10.3	30.6	956	22.2	695	3.5
	480	33	3.0	11.0	38.4	1,164	22.6	685	3.7
T. R.	0	42	3.0	14.0	22.0	524	22.0	524	3.2
7/2—56	260	35	3.0	11.7	32.4	926	23.3	665	3.4
	480	43	3.5	12.3	46.1	1,072	25.5	593	3.4
R. J.	0	39	2.8	13.9	24.3	623	24.3	623	3.8
2/12—55 ...	260	30	2.5	12.0	31.8	1,060	24.0	799	3.5
	480	37	2.5	14.8	42.7	1,154	26.4	679	3.8
R. J.	0	30	2.6	11.5	20.8	693	20.8	693	2.6
1/2—56	260	44	3.5	12.8	33.9	771	22.4	510	3.4
	480	45	3.9	13.2	45.2	1,004	23.8	529	3.6
L. Å.	0	36	2.3	15.6	21.6	600	21.6	600	2.5
19/3—56 ...	260	45	3.0	15.2	36.8	820	25.1	560	2.9
	480	49	3.3	14.8	49.9	1,020	25.4	540	3.3
L. Å.	0	44	2.6	16.9	21.7	495	21.6	495	2.5
21/3—56 ...	260	49	3.1	15.7	34.8	710	22.1	470	2.8
	480	49	3.1	15.7	47.8	975	24.3	535	3.0
G. L.	0	32	2.5	12.8	19.8	620	19.8	620	2.7
20/3—56 ...	260	38	2.8	13.6	32.1	845	22.2	585	3.3
	480	42	2.9	14.5	43.6	1,040	23.4	600	3.9
G. L.	0	31	2.4	12.9	19.2	618	19.2	618	2.7
20/5—56 ...	260	33	2.4	13.8	29.5	870	20.9	610	3.1
	480	40	2.7	14.8	41.9	1,050	22.7	610	3.7
T. R.	0	35	3.1	11.4	22.8	655	22.8	655	3.3
19/3—56 ...	260	28	3.1	9.0	29.0	1,035	21.7	775	3.8
	480	25	3.2	7.7	34.8	1,400	22.8	960	4.7

L.

with 5 normal subjects.

f_1 Fraction volume L		w_1	f_2 Fraction volume L		w_2	Average no. of breaths N_2 molecules remain			Vol. O_2 wash-out/ FRA +extr. D. S.	Calcul. D. S. ml.
						actual	ideal	delay in %		
0.34	0.88	0.946	0.66	1.72	0.807	9.7	6.7	46	9.0	186
0.26	0.93	0.978	0.66	1.85	0.899	18.7	7.2	160	9.4	92
0.77	2.39	0.950	0.23	0.71	0.814	16.7	11.8	41	9.2	162
0.64	1.73	0.953	0.36	0.97	0.809	15.7	10.1	53	8.6	85
0.95	3.14	0.932	0.05	0.17	0.646	14.0	11.6	21	7.2	115
0.93	3.72	0.950	0.07	0.28	0.728	18.6	14.0	33	7.2	168
0.86	3.78	0.887	0.14	0.62	0.660	8.0	6.5	23	5.3	70
1.00	3.90	0.890				9.2	9.2	0	5.8	210
1.00	4.50	0.917				10.9	10.9	0	5.8	236
0.51	1.73	0.928	0.49	1.67	0.796	9.5	7.1	35	6.7	130
0.75	3.00	0.904	0.25	1.00	0.839	9.4	8.8	6	5.8	179
1.00	4.30	0.880				8.3	8.3	0	6.0	217
0.91	2.73	0.905	0.09	0.27	0.458	9.8	6.0	62	7.4	0
0.93	3.26	0.888	0.07	0.24	0.603	8.5	4.9	72	6.3	131
0.90	3.33	0.965	0.10	0.37	0.605	8.8	6.8	30	6.1	323
0.81	2.56	0.914	0.19	0.61	0.756	10.2	8.3	22	6.9	95
0.88	2.99	0.892	0.12	0.41	0.769	8.6	8.0	8	6.9	132
1.00	3.40	0.909				10.9	10.9	0	7.5	225
0.79	3.00	0.917	0.21	0.80	0.790	10.5	8.8	19	6.4	145
1.00	3.50	0.879				8.3	8.3	0	6.9	315
1.00	3.80	0.910				11.1	11.1	0	6.9	334
0.84	2.18	0.909	0.16	0.42	0.643	8.5	6.4	32	8.0	116
1.00	3.40	0.895				11.9	11.9	0	6.6	202
1.00	3.60	0.916				11.9	11.9	0	6.6	199
0.64	1.57	0.918	0.36	0.88	0.777	9.4	7.3	29	8.8	200
0.58	1.71	0.939	0.42	1.23	0.833	12.0	9.2	30	8.5	200
0.74	2.46	0.938	0.26	0.87	0.828	13.4	10.8	24	7.6	200
0.89	2.25	0.918	0.11	0.28	0.570	13.4	7.2	86	8.5	90
1.00	2.75	0.918				12.2	12.2	0	8.0	200
1.00	3.02	0.920				12.5	12.5	0	8.0	245
0.52	1.40	0.914	0.48	1.30	0.786	8.2	6.6	24	7.3	130
0.52	1.71	0.927	0.48	1.58	0.843	10.2	8.7	17	6.7	190
0.75	2.91	0.921	0.25	0.97	0.857	11.2	10.5	7	6.0	210
0.84	2.25	0.890	0.16	0.43	0.639	8.0	6.1	31	7.2	90
0.93	2.92	0.901	0.07	0.22	0.487	9.5	6.8	40	6.7	110
1.00	3.67	0.900				10.0	10.0	0	6.2	210
0.93	3.11	0.901	0.07	0.23	0.877	10.0	9.9	1	6.8	130
1.00	3.28	0.863				7.3	7.3	0	5.7	205
0.91	4.27	0.872	0.09	0.42	0.650	7.4	6.5	14	4.9	250

Table

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Subject	D. S. added ml	Total no. of breaths	Wash-out time min.	Resp. freq. /min.	Total vent. L	Tidal air ml.	Vol. O_2 wash-out lungs L	Tidal vol. wash-out lungs ml	FRA +extr. D. S. L
T. R.	0	34	2.9	11.7	21.5	597	21.5	597	3.1
20/3—56 ...	260	36	3.2	11.3	30.7	852	21.3	592	3.7
	480	40	3.7	10.8	42.1	1,053	22.9	613	4.3
B. W.	0	33	3.2	10.3	23.9	725	23.9	725	3.6
20/3—56 ...	260	40	3.6	11.1	35.1	880	24.7	620	3.8
	480	40	3.7	10.8	43.5	1,090	24.3	650	4.3

Column (2): extra dead space (0, 260 or 480 ml). Column (3): total number of (litres BTPS). Column (8): Total ventilation minus resp. frequency times extra of the subject. Column (9): Tidal volume minus volume of extra dead space. Columns ments. Column (20): lung clearance index calculated for the lungs of the subject, is the volume of oxygen which reaches the lungs + airways of the subject (column

system (extra dead space included) and secondly for the lungs only by subtracting the ventilation of the extra dead space. The efficiency of the nitrogen wash-out of the total system is diminished: without extra dead space 7.4 litres are ventilated for the wash-out of one litre of the total system; after addition of an extra dead space 12.2 L are ventilated per litre. The efficiency of the lungs proper is not impaired, as shown by the values after subtraction of the ventilation of the extra dead space. The average values even show a slight increase in efficiency: the significance of this increase is, however, doubtful. The subject's dead space, calculated from the "ideal dilution factor", shows a significant increase when extra dead space is added.

Figs. 1—5 illustrate these findings. Fig. 1 demonstrates the change in the form of the semilogarithmic plot after addition of a dead space. Without extra dead space there is a two-fraction ventilation, with a delay percentage of 35 %. With 260 ml extra dead space (not shown in Fig. 1; see Table I) the delay percentage decreases to 6 %, and with 480 ml extra dead space (Fig. 1) the semilog plot is rectilinear, corresponding to a delay percentage of 0. Fig. 2 gives the delay percentages for our 15 experiments. Without extra dead space the delay percentage is never zero, the lowest value being 1 %; with 260 ml extra dead space it is zero in 5 out of 15 experiments and with 480 ml the delay percentage is zero in 8 out of 15 experiments. Only one experiment forms a clear

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f_1 Fraction volume L		w_1	f_2 Fraction volume L		w_2	Average no. of breaths N_2 molecules remain			Vol. O_2 wash-out/ FRA +extr. D. S.	Calcul. D. S. ml.
						actual	ideal	delay in %		
0.96	2.96	0.892	0.04	0.12	0.262	9.0	5.4	67	7.0	200
0.96	3.50	0.896	0.04	0.20	0.685	9.3	8.9	5	5.8	157
1.00	4.28	0.902				10.2	10.2	0	5.4	173
0.39	1.39	0.933	0.61	2.17	0.809	9.1	6.8	34	6.7	140
0.74	2.83	0.921	0.26	0.99	0.816	10.8	9.5	14	6.5	155
0.52	2.23	0.932	0.48	2.05	0.836	10.5	8.6	22	5.7	160

breaths during wash-out test. Column (6): total ventilation during wash-out test dead space, *i. e.* the volume of inspired oxygen which reached the lungs + airways (11) to (16) inclusive: relative volumes (f) and dilution factors (w) of lung compart- subtracting the ventilation of the extra dead space. The numerator of the index (8)); the denominator is the post-inspiratory lung volume of the subject.

exception, with an increase in delay percentage to 160 % with 260 ml extra dead space.

Fig. 3 demonstrates the relationship between dead space and tidal volumes in these 15 experiments. The regression equation of dead space on tidal volume shows that the dead space is on the average very near 20 % of the tidal volume. A comparison of our values with those obtained by SHEPARD et al. (1955) shows a fairly good agreement between the dead space as a percentage of the tidal volume in both studies, the average $V_D/V_T \times 100$ being 18 in SHEPARD's study (Fig. 4). It might be asked whether the high degree of correlation between dead space volumes and tidal volumes is coincidental. In most experiments, the post-inspiratory lung volume increased as well as the tidal volume when extra dead space was added. It might be that the increase in dead space after addition of extra dead space was in reality due to the increase in the post-inspiratory lung volume. Fig. 5 shows the relation between calculated dead space volumes and post-inspiratory lung volumes. There is a positive correlation ($r = + 0.66$), but of a lesser degree than the correlation between dead space and tidal volume ($r = + 0.82$).

Discussion.

In our study on hyperventilation during exercise we found no consistent changes in either the lung clearance index or the pulmonary nitrogen clearance delay percentage. In the

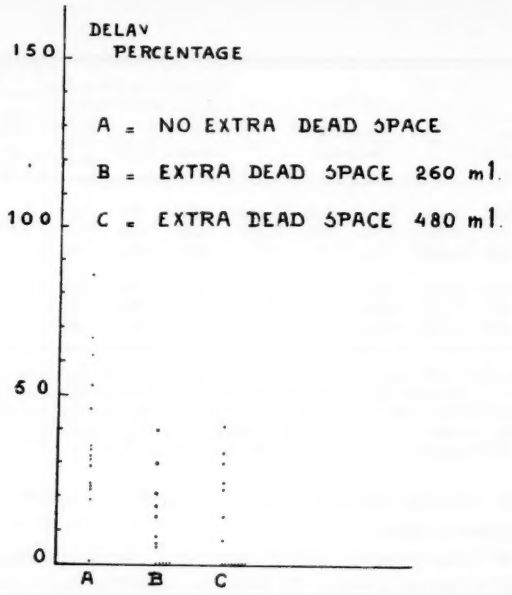

Fig. 2. Delay percentages in 15 experiments at rest (column A), with 260 ml extra dead space (column B) and with 480 ml extra dead space (column C).

Fig. 3. Regression of dead space (y) on tidal volume (x).

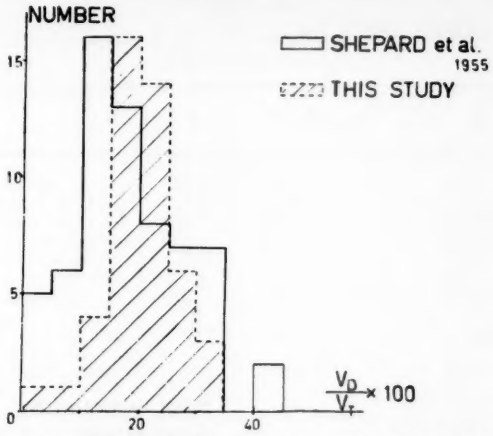

Fig. 4. Frequency-diagram of V_D/V_T ratios in experiments of SHEPARD et al. (1955) and in this study.

present study, however, the lung clearance index did not change appreciably, whereas the pulmonary nitrogen clearance delay percentage decreased significantly. How are these discrepant findings in the two forms of hyperventilation to be explained? The first question is: why do we find in one form of hyperventilation (by adding dead space) an increase in uniformity of ventilation, and no change in uniformity in the other form of hyperventilation (exercise)? Uniformity of ventilation is studied with our methods as uniformity of distribution of inspired oxygen, and when we add an extra dead space the first part of the inspiration is only dealing with inspiration of dead space gas into the lungs. It might be that the improvement in uniformity with extra dead space is only apparent, and that in reality the hypoventilated areas are being filled with dead space gas in the first part of inspiration while the hyperventilated areas are being filled with oxygen in the later part of inspiration. This hypothesis, however, assumes a rather high degree of preferential filling of hypoventilated areas with dead space gas. That there may be such a mechanism has been pointed out by RAUWERDA (1946) and by FOWLER (1952): "sequential ventilation" or "first-in, last-out principle". We think that this mechanism cannot be of major importance in normal subjects. This opinion is based upon the results of LUNDIN (1955) who demonstrated that in normal subjects the semilog plots of end-tidal nitrogen concentration

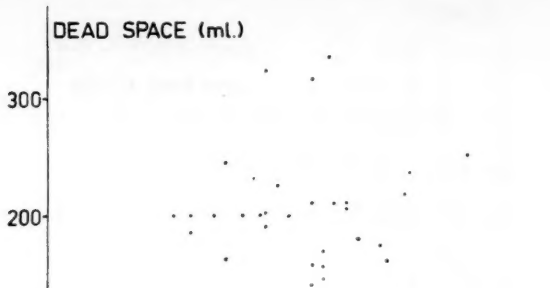

Fig. 5. Relation between calculated dead space volumes and post-inspiratory lung volumes. Correlation coefficient $r = +0.66$. See text.

during a wash-out experiment run parallel to semilog plots of simultaneously recorded amounts of nitrogen washed out per breath. Without going into the mathematical aspects of this question we wish to point out that this finding makes a substantial amount of preferential filling of hypoventilated areas with dead space gas improbable.

A second possibility is a real change in the ventilation ratios of the different lung areas when a dead space is added, with more equal ventilation in all lung parts as a result. While there may, theoretically, be such changes they have not been demonstrated directly. In patients with abnormally high and unevenly distributed airway resistances and with locally different lung compliances (OTIS, MCKERROW, BARTLETT, MEAD, MCILROY, SELVERSTONE and RADFORD 1956) it would be possible to visualize opening of partly obstructed small airways due to the higher end-inspiratory lung volume, which could give rise to more uniform ventilation; in the normal subjects we studied such effects are highly improbable.

In our opinion, a third explanation of our findings is the most probable one. If the alveolar gas concentrations during nitrogen wash-out in a model experiment are calculated, assuming even mixing within the lung spaces and assuming proportional distribution of dead space gas over the lung fractions, the influence of

Table II.

Average values of the 15 experiments from Table I.
Extra dead space.

	0	260 ml	480 ml
Resp. freq./min.	12.9	12.4	12.9
Funct. Res. Vol. (L)	3.1	3.1	3.3
Tidal volume (ml) ...	620	870	1.080
Tid. vol.—extra d. s. (ml)	620	610	600
Act. ave. no. breaths.	9.9	10.7	11.5
Ideal ave. no. breaths	7.3	8.8	10.3
Delay percentage (FOWLER)	38	25 ($p = 0.041$)	11 ($p = 0.00046$)
Wash-out vol. per litre of funct. res. vol. (1) for the total system: (L/L)	7.4	9.9	12.2
(2) after subtraction of d.s. ventilation:(L/L)	7.4	6.9	6.6
Dead space of subject (ml)	120	173 ($p = 0.006$)	221 ($p = 0.006$)

The wash-out volumes per litre of functional residual volume, calculated for the total system (1) have not been given in Table I; the same wash-out volume calculated after deduction of dead space ventilation (2) corresponds to the values in Table I, column (20). The p values refer to the probability that the observed differences from the values found without extra dead space are due to chance (p calculated from the 'sign test' of DIXON and MOOD, 1946).

the dead space upon the mixing process is easily understood. This is best illustrated by a semi-quantitative example. If we assume that two lung compartments are being washed out, one of them with twice as large a tidal volume as the other, starting from a hypothetical concentration of 1, we can find a course of the wash-out process in the two fractions as shown in Table III.

After each expiration the dead space remains filled with a mixture of gas from the two fractions; the concentration of this mixture is given in the 3rd column. Of course this concentration is always somewhere between the alveolar concentrations in the two fractions. This gas from the dead space is the first to be inspired in the next breathing cycle into both fractions. As its concentration is always higher than that in the hyperventilated fraction, the inspiration of dead space gas into this fraction tends to decelerate the wash-out process; conversely, the inspiration of dead space gas into the hypoventilated fraction speeds up the wash-out in this compartment. Thus, the mixing of gas from different lung fractions in the dead space is in itself a factor which contributes to more equal alveolar gas concentrations throughout

Table III.

The nitrogen concentrations during the first ten breaths of a wash-out procedure in a theoretical two-fraction system, assuming all non-uniformity to be due to regional inequalities of ventilation.

No. of resp.	Conc. in hypervent. fraction	Conc. at end-exp. in d. s.	Conc. in hypovent. fraction
0 ...	1	1	1
1 ...	0.833	0.860	0.905
2 ...	0.696	0.736	0.817
3 ...	0.583	0.634	0.735
4 ...	0.490	0.547	0.660
5 ...	0.413	0.473	0.592
6 ...	0.349	0.409	0.530
7 ...	0.296	0.355	0.474
8 ...	0.252	0.309	0.423
9 ...	0.215	0.269	0.377
10 ...	0.184	0.235	0.336

Arbitrary measures were used for all parameters of the model: nitrogen concentration in all parts 1 at the start; volume of both fractions 36; tidal volume of first fraction (column (2)) 12; tidal volume of second fraction (column (4)) 6; dead space 6. Dead space gas was considered to be ventilated proportional to tidal volumes of the fractions. See text.

the lungs. This effect is easily demonstrated in calculations where normal assumptions about the value of the dead space volume are made, and it increases with increasing values of the dead space. In our opinion this is the most probable explanation of the higher degree of uniformity of ventilation when extra dead space is added to breathing.

Notwithstanding the better distribution, the nitrogen clearance of the lungs is not improved significantly. The most probable reason for this is the increase in dead space of the subject which cancels out the improvement in clearance efficiency caused by better distribution. The problem of why the dead space volumes increase with higher tidal volumes is very complicated. Such an increase is regularly found with methods measuring the physiological dead space (ROSSIER and BÜHLMANN 1955), but its occurrence with our method is a little unexpected. Any method using a mixing criterion of dead space would be expected to measure anatomical rather than physiological dead space. This question is difficult to explain at present; however, the reason why the physiological dead space is proportional to tidal volume is equally difficult to decide.

The results of this study on the efficiency of ventilation when

extra dead space is added to breathing confirm the results of our earlier experiments, *i. e.* that hyperventilation does not entail any significant amelioration of ventilatory efficiency.

Summary.

Hyperventilation due to extra dead space did not cause any significant improvement in ventilatory efficiency as measured in nitrogen wash-out experiments during oxygen breathing. In the experiments with extra dead space a significant increase in degree of uniformity of ventilation could be demonstrated. The subject's dead space increased, and the over-all efficiency remained constant. The efficiency-improving effect of a better distribution and the efficiency-deteriorating effect of a larger dead space probably cancelled out. The better distribution is most probably explained by the effect of mixing of gas from different lung fractions in the large dead space. The experiments were performed with a nitrogen meter.

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Species Differences of Clotting Factors in Ox, Dog, Horse, and Man.

Proaccelerin and Accelerin.

By

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Proaccelerin (factor V, labile factor, plasma Ac-globulin) plays an important role in the kinetics of the coagulation process. Its influence covers both the speed of formation and the yield of thrombin. During coagulation proaccelerin is converted by thrombin to a more active product called accelerin (serum Ac-globulin). In this way proaccelerin becomes the key substance in the pseudo-autocatalytic mechanism of thrombin formation.

Preparations of proaccelerin and accelerin from different species are frequently used in coagulation studies. In spite of this, species differences have received little attention. It is interesting that FANTL and NANCE (1946) obtained evidence for their prothrombin accelerator (which is identical to proaccelerin) from experiments with dilution of human plasma in adsorbed rabbit, dog, guinea pig, and human plasma. They found decreasing activity of plasma in the order mentioned, and we now know that this is in full accord with the concentration of proaccelerin in the plasmas of these species. Other data available concerning species differences are quantitative estimations of proaccelerin in various species in Quick's and Seegers' laboratories. Concerning qualitative differences, only one statement has been found, namely that proaccelerin from bovine plasma is more stable than human proaccelerin. This was first noted by OWREN (1947).

The purpose of the present work was to study species differences of proaccelerin and accelerin both quantitatively and qualitatively in beef, dog, and horse plasma as compared with human plasma.

Materials.

Proaccelerin deficient plasma. This was made from citrated blood obtained from Owren's patient with parahemophilia, and stored in small aliquots at — 20° C. The plasma, thought to be free of proaccelerin, has a normal content of prothrombin and proconvertin.

Saline brain thromboplastin was made from brain according to Owren (1947).

Thrombin. Thrombin from different species was made in the following way: Citrated plasma was diluted ten times with cold distilled water and the pH adjusted with N/2 hydrochloric acid to 5.4. After one hour in the cold, it was centrifuged and the supernatant discarded. The precipitate was dissolved in veronal buffer, homologous thromboplastin and calcium chloride to 2.5 mM were added and the mixture was placed in the water bath at 37° C for 30 minutes. The fibrin formed was removed, and after complete conversion the thrombin solution was dialyzed against 0.9 per cent saline overnight. It was then centrifuged in the cold for one hour at 40,000 rpm and the supernatant frozen in the liquid state at — 20° C in small aliquots.

Veronal buffer was made as described by Owren (1947), pH 7.35 and ionic strength 0.154.

Citrated dilution fluid I was made by mixing one part of 3.13 per cent sodium citrate dihydrate and five parts of 0.9 per cent saline. The citrate concentration of this fluid is similar to that of plasma from citrated blood.

Citrated dilution fluid II.

Veronal buffer	200 ml
Sodium citrate (25.66 mM)	200 »
Sodium chloride 0.9 per cent	600 »

Anticoagulants. Sodium citrate dihydrate, 3.13 per cent, or potassium oxalate monohydrate, 2.5 per cent, were used. It was noted that with these anticoagulant solutions hemolysis often occurred in dog blood. However, if the pH of the solutions was reduced to 6.0—6.5, with N/1 HCl, and the blood was drawn from fasting individuals no hemolysis was seen.

Dialysing casings. Cellophane casings manufactured by the Visking Corporation, Chicago, were used.

Barium sulphate (Baker) analytical grade was used for adsorption.

Methods.

Proaccelerin assay. The one-stage method of Owren (1947) was used, with his patient's plasma as substrate plasma. The clotting times were converted to per cent activity from a correlation graph which had been made by diluting a mixture of ten normal human plasmas. The

Table 1.

The concentration of proaccelerin in human, dog, beef, and horse plasma.

Human	Dog	Beef	Horse
92	890	450	260
78	780	500	340
120	1,050	650	350
105	980	620	240
92	1,040	390	300
100	680	460	310
135	970	540	400
90	1,100	550	220
88	840	620	230
98	760	420	280
$\bar{m}$ 100	910	520	290
Range 78—135	680—1,100	390—650	220—400

The plasma was obtained from citrated blood by centrifugation at 2,500 rpm for 30 minutes in the cold, and the assay performed within two hours after withdrawal of the blood.

1/10 dilution of citrated or oxalated plasma was arbitrarily taken as 100 per cent. Thus, the figures obtained for other species are relative to the normal human plasma level. It is stressed that the values obtained are in terms of activity and do not bear any relation to quantitation on the basis of weight.

Accelerin assay. The method used was the same as for proaccelerin. The justification of this is based on the finding that the correlation graph showed the same slope for both proaccelerin and accelerin.

Accelerin capacity. Assay of accelerin capacity of proaccelerin in plasma was carried out as outlined by HJORT (1957) for purified preparations, and is described in the chapter dealing with accelerin capacity.

Measurements of pH were made electrometrically with a Radiometer pH-meter, Copenhagen.

Collection of blood. Ox and horse blood were collected by puncture of the jugular vein, dog blood by puncture of the cephalic or saphenic vein, and human blood by puncture of the antecubital vein. The blood was allowed to flow freely into a graduated tube containing the anticoagulant. After centrifugation the hematocrit was read from the scale.

Experiments.

I. *Proaccelerin levels in beef, dog, horse, and human plasma.* Proaccelerin activity was assayed in ten individuals in each species, the plasmas being diluted as follows: beef 1/200, dog 1/500, horse 1/100, and human 1/50. The results are shown in table 1. Proaccelerin activity varied from one species to another. Further,

Table 2.

Performance of the accelerin capacity test. (Human plasma.)

A. Proaccelerin.						
Dilution 1/50 Coagulation time 37.5 secs. corresponding to 115 per cent proaccelerin						
B. Accelerin formation after addition of thrombin						
Dilution	1/500					
Incubation time, secs	30	60	90	120	180	240
Coagulation time, secs	36.0	32.1	31.0	30.5	30.8	31.2
Accelerin per cent	1,300	1,950	2,200	2,400	2,250	2,100
Accelerin capacity	2,400/115 = 21					

Table 3.

The accelerin capacity in human, dog, beef, and horse plasma.

Human	Dog	Beef	Horse
20	9	40	14
21	8	37	13
21	9	44	15
25	7	42	14
24	6	38	13
22	7	37	12
25	8	38	15
20	5	42	12
20	7	44	13
19	8	36	15
$\bar{m}$ 21.7	7.4	40	13.6
Range 19—25	5—9	36—44	12—15

The plasma was obtained from citrated blood by centrifugation at 2,500 rpm for 30 minutes in the cold, and assayed within two hours after withdrawal of the blood.

there are also great variations among individuals within one species even after correction for the hematocrit. This might be due partly to inaccuracy of the method. However, reproducible results were found in repeated assays with one plasma. Possibly, some proaccelerin might also be activated to accelerin in the short interval between withdrawal and the assay procedure. Experiments with the accelerin capacity test, however, failed to demon-

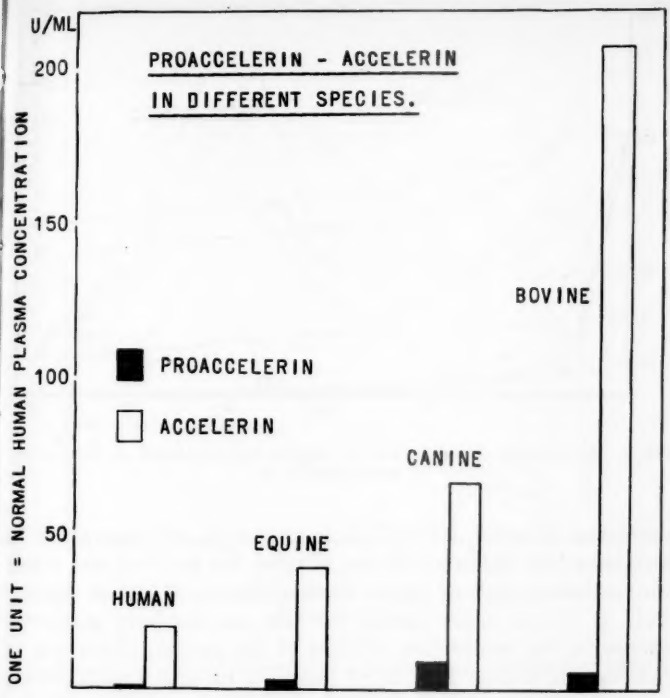

Fig. 1. The relation between actual (proaccelerin) and potential (accelerin) accelerator activity in plasmas from different species.

strate any activation. It is therefore believed that the individual variations are of this magnitude.

II. *Accelerin capacity of proaccelerin in different species.* "Accelerin capacity" is defined as the ratio between the amount of accelerator activity found after, and the amount found before addition of thrombin. The amount of thrombin added must be great enough to secure complete conversion of the proaccelerin, while on the other hand it must not influence the test system. In the following experiments the accelerin capacity was estimated in plasmas from the species studied. The test was carried out in the following way. Beef plasma: Preliminary dilution 1/100 in dilution fluid I. Incubation mixture: 1.7 ml dilution fluid I + 0.2 ml of preliminary dilution + 0.1 ml of thrombin solution, the con-

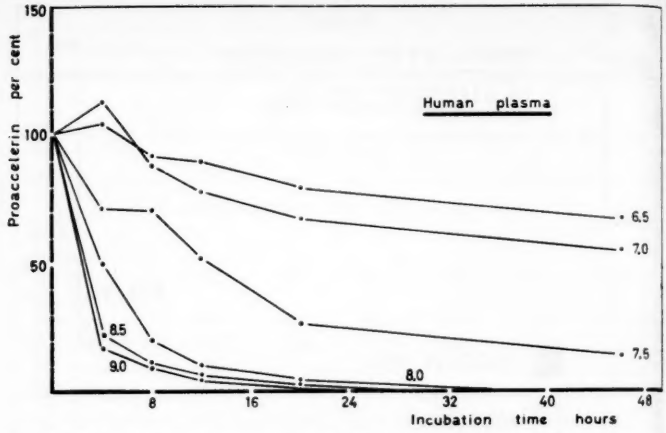

Fig. 2. The stability of proaccelerin in citrated human plasma at various pH. Temperature 37° C.

centration of which was 4 NIH units per ml. At intervals of 30 seconds 0.2 ml of the incubation mixture was removed and tested for accelerator activity after a further dilution of 1/10 in dilution fluid II. In the other species the test was similarly performed except for the preliminary dilution of the plasma, which was as follows: dog plasma 1/20, horse plasma 1/10, and human plasma 1/10. Usually the maximal activity was found after two minutes with this plasma dilution and thrombin concentration. The thrombin used was of bovine origin for all plasmas. In experiments with homologous thrombin it was noted that the origin of thrombin was without influence on the results. The amount of thrombin used was probably above the level at which species differences can be revealed. As an example, the complete figures from one test carried out in human plasma are given in table 2. Proaccelerin was estimated just before the start of incubation. The results of ten plasmas from each species are given in table 3. Marked differences between species are seen also in respect to accelerin capacity. However, when compared with the results in table 1 it is seen that no parallel exists between proaccelerin concentration and accelerin capacity. This is more clearly visualized in figure 1, which shows both the *actual* accelerator capacity (proaccelerin) and the *potential* capacity (accelerin) of plasmas from the species concerned. Special emphasis is laid upon the difference between

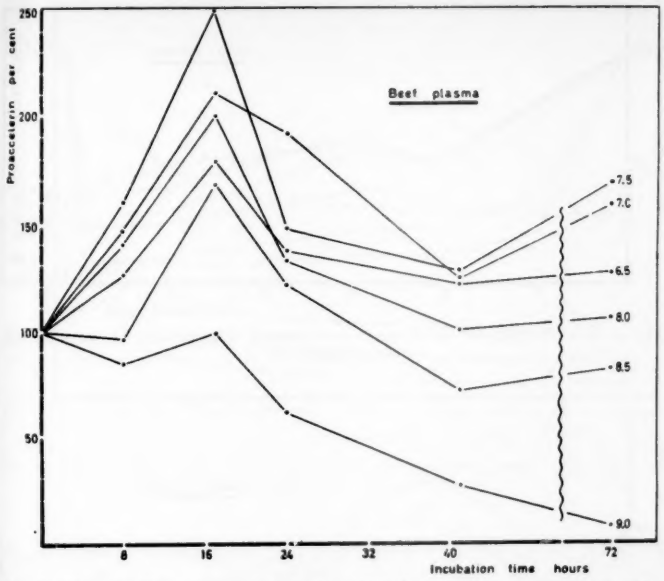

Fig. 3. The stability of proaccelerin in citrated beef plasma at various pH. Temperature 37° C.

beef and dog plasma. In spite of a lower actual accelerator capacity in beef plasma, its potential capacity is much higher.

III. *Species differences in regard to the physico-chemical properties of proaccelerin and accelerin.*

1. *Stability of proaccelerin.* In the stability experiments reported in this paper citrated plasma was used. After withdrawal, the blood was immediately centrifuged for 30 minutes at 2,500 rpm to remove most of the platelets and the red blood cells. The plasma was dialyzed against dilution fluid I at 4° C for 24 hours. After the pH had been adjusted with N/2 HCl or N/2 NaOH as indicated in the figures, the incubation was started. The plasma was stored in 10 ml tubes cleaned in chromate-sulphuric acid. A temperature of 37° C was selected after preliminary experiments. In the presentation of results, the preincubation values were taken as 100 per cent in each species. The stability was judged from the slope of the curves, disregarding the absolute amounts of activity which were lost during incubation. From the curves in figures

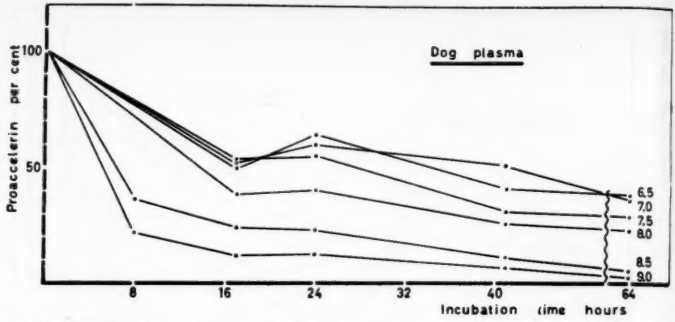

Fig. 4. The stability of proaccelerin in citrated dog plasma at various pH. Temperature 37° C.

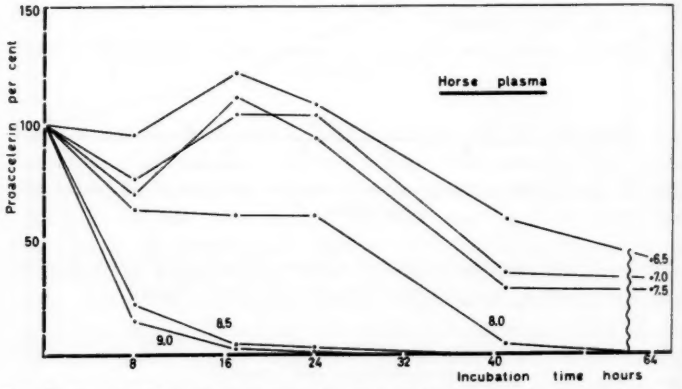

Fig. 5. The stability of proaccelerin in citrated horse plasma at various pH. Temperature 37° C.

2, 3, 4, and 5 it appears that pH is of decisive importance for the stability of proaccelerin. The higher the pH, the less the stability. Secondly, the difference in stability among species is evident. This is especially obvious for the bovine species versus the other ones, proaccelerin of bovine origin being the most stable. The differences between the other species are less clear, probably proaccelerin in dog plasma was more stable than proaccelerin in horse and human plasma, which seemed to behave similarly. Thirdly, the curves show that at least some proaccelerin was converted to accelerin since temporary increase in activity was

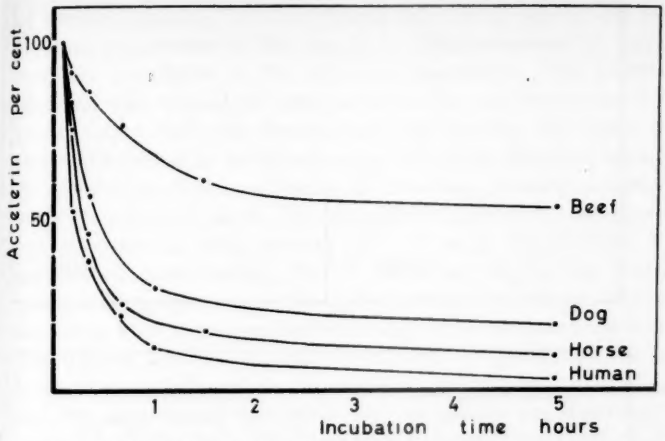

Fig. 6. The stability of accelerin in citrated, defibrinated human, dog, beef, and horse plasma. pH: 7.8, temperature 37° C.

found during incubation. This activation occurred especially in the lower pH range, gradually diminishing with increasing pH. The activation was encountered in all species studied, but to variable degrees. The bovine and canine species represented the extremes, the first showing marked activation, the latter just measurable activation.

Experiments at 56° C showed the same stability pattern among the species, but at this temperature no activation was observed. In oxalated plasma at 37° C the stability was much less, but followed the same species pattern as for citrated plasma, and activation also occurred. In ion exchange resin plasma proaccelerin was more stable than when any other anticoagulant was used, and in oxalated, adsorbed, dialyzed, and recalcified plasma, proaccelerin was even more stable.

2. *Stability of accelerin.* Citrated dialyzed beef, dog, horse, and human plasma were activated with thrombin. The thrombin was added repeatedly in amounts of one unit per ml plasma at intervals of two minutes, and the fibrin removed each time. At least two portions of thrombin were added after the last trace of fibrin was removed. In all about 10 thrombin units per ml plasma were used in order to secure complete conversion of proaccelerin. This was checked with the accelerin capacity test. The measurements

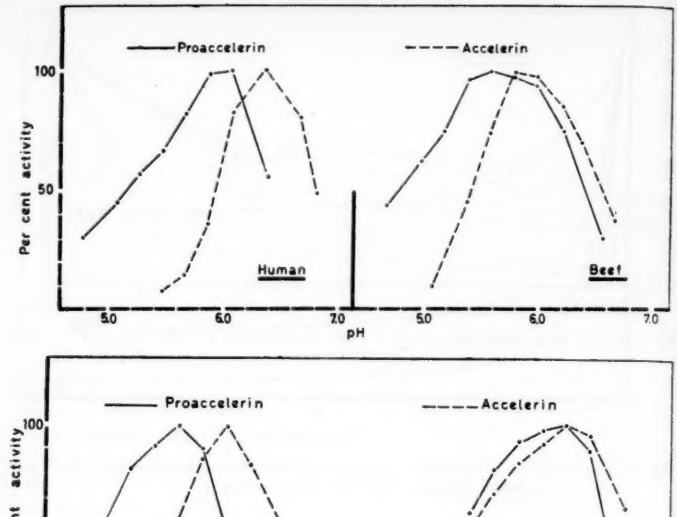

Fig. 7 a. Acid precipitation of proaccelerin and accelerin in diluted human and beef plasma.

Fig. 7 b. Acid precipitation of proaccelerin and accelerin in diluted horse and dog plasma.

started 5 minutes after the last addition of thrombin and this value was taken as 100 per cent. At this time no influence on the test system from the remaining thrombin was found. From figure 6 it is seen that accelerin was found to be present in all species. Immediately after defibrination it disappeared rapidly, then at a slower rate and finally the activity changed very slowly. The species stability pattern is the same as for proaccelerin. As with proaccelerin, the stability was much less when oxalate was used as the anticoagulant. In ordinary serum, stability was greater than in serum with either citrate or oxalate added.

3. *Acid precipitation of proaccelerin and accelerin.* Oxalated beef, dog, and human plasmas were adsorbed with 100 mg per ml

of barium sulphate. In horse plasma only 25 mg per ml was used because proaccelerin in this species is readily adsorbed by barium sulphate as shown in the following paragraph. The adsorbed plasmas were divided into two parts, the one used for proaccelerin precipitation, the other for accelerin precipitation. The latter was thus defibrinated as described above and left in the water bath for 30 minutes to allow inactivation of thrombin. It was then immediately cooled in ice water. The precipitation procedure was carried out similarly in both portions and ice water temperature was maintained until testing. Before adjusting pH to the desired values with one per cent acetic acid, four ml of plasma were diluted ten times with precooled distilled water in 50 ml centrifuge tubes. The diluted acidified plasma was allowed to precipitate for one hour, then centrifuged for 10 minutes at 2,000 rpm in the cold and the supernatant discarded. The precipitate was dissolved in veronal buffer to the original plasma volume and tested immediately for accelerator activity. The results are shown in figures 7 a and b. First it is seen that the isoelectric point (maximal precipitation) under these conditions is at a higher pH than previously thought. Secondly, the isoelectric point for accelerin is higher than for proaccelerin. An exception to this is seen in the dog, where no significant difference was found. Concerning the isoelectric point for different species, it is higher in the dog plasma than in the others. On the basis of the small differences found among beef, horse, and human proaccelerin or accelerin, one cannot conclude that the isoelectric points differ, since variations of equal magnitude were found in different experiments.

4. *Adsorption of proaccelerin and accelerin onto barium sulphate.* It was noted in the precipitation experiments that horse proaccelerin was adsorbed to a marked degree, thereby causing difficulties in carrying out the experiments. This revealed a species difference in adsorbability, and the adsorption experiments reported in table 4 were therefore done. Proaccelerin in oxalated plasma and accelerin in oxalated plasma defibrinated with thrombin was measured before and after adsorption with 100 mg barium sulphate per ml plasma. It is seen that little proaccelerin was adsorbed from beef, dog, and human plasma. In horse plasma between 70 and 80 per cent, sometimes more was adsorbed. Accelerin, on the other hand, was well adsorbed in all species. In the table the units of proaccelerin or accelerin adsorbed are given to show that the

Table 4.

Adsorption onto barium sulphate of proaccelerin and accelerin in different species.

Species	Proaccelerin (native plasma)				Accelerin (defibrinated plasma)			
	Before ads.	After ads.	% ads.	U. ads.	Before ads.	After ads.	% ads.	U. ads.
Human ..	100	75	25	25	80	2	98	78
Dog	800	720	10	80	1,500	20	98	1,480
Beef	500	390	20	110	2,700	225	92	2,475
Horse ...	260	70	75	180	190	3	98	185

The adsorption was made with barium sulphate (Baker) 100 mg per ml plasma, at room temperature for 10 minutes.

adsorption was not dependent on quantitative, but on qualitative differences.

Discussion.

In 1943, Quick showed that dog plasma was richer in component A of prothrombin, which later was shown to be identical to proaccelerin, than was human plasma. Quick and Stefanini (1948) stated that dog plasma contained ten times more proaccelerin than human plasma, and Murphy and Seegers (1948) found 12 and 9 times as much in dog and beef plasma, respectively, as in human plasma. These values are therefore somewhat greater than those found in this study, indicating that the two methods do not give identical results. Apparently, estimations on horse plasma have not been published.

The experiments on accelerin capacity in plasma might be criticized for two reasons. Firstly, the formation of accelerin is followed by an inactivation (see figure 6), much like the inactivation of thrombin when formed in plasma. A true peak value cannot, therefore, be obtained. In high plasma dilutions however, the activation curve did not change appreciably during some time after the maximal activity was reached (table 2), indicating that almost all activity was recorded. Secondly, some accelerin is adsorbed onto fibrin during coagulation according to our own experiments. The true accelerin capacity should thus be somewhat higher than judged from the test, but none of these objections is thought to invalidate the observed differences among

species. The results with the accelerin capacity test indicate that proaccelerin is of different chemical organization in different species. Further, the difference in actual and potential activity especially between beef and dog proaccelerin raises the question of whether proaccelerin is active or inactive before being acted upon by thrombin. This problem is being studied more closely.

Concerning the stability experiments with proaccelerin and accelerin, it is essential to standardize the conditions properly, since FAHEY, WARE and SEEGERS (1948) showed that stability in plasma was influenced by the number of platelets, by the type and concentration of anticoagulant, and indirectly by the glassware. QUICK and STEFANINI (1948) concluded that the decrease in activity was related to the decalcification, and caused by oxidation, since samples protected from air contact showed greater stability. None of these authors, however, have taken the pH into account, a most important factor determining the stability of proteins. OWREN (1947) tested the resistance of proaccelerin in human plasma against acid and alkali and found that at pH 10.5 it was inactivated in less than one hour. The same pH was used by MUNRO and MUNRO (1947) for inactivating proaccelerin in dog plasma. In the experiments of this study the carbonic acid-bicarbonate buffer system was removed from plasma by dialysis in order to obtain proper control of the pH, which can only be achieved when either the volatile component or the whole buffer system is removed.

The results of the stability experiments show that pH is of outstanding importance for proaccelerin preservation. In view of this we propose that Quick's finding was due to differences in the pH of oil-covered and non-covered plasmas. This explanation is further substantiated by our finding that in dialyzed plasmas no difference in stability was found between oil-covered and non-covered samples. In a non-covered sample of plasma which has not been dialyzed carbon dioxide can freely escape, thereby causing a rise in pH with a consequently lower stability.

The observed activation during incubation indicates that proaccelerin at least partly is inactivated after first being activated to accelerin. However, it cannot be decided whether this is the main way of inactivation. The statement of FAHEY, WARE and SEEGERS (1948) that proaccelerin in citrated plasma is stable does not fit with these findings, which indicate only a pseudostability. Due to the activation of proaccelerin the activity measured might keep

fairly constant over a period of time, although the absolute amount of accelerator activity decreases.

Concerning the stability of accelerin, several factors might intervene, *i. e.* antiaccelerin (OWREN 1947), destruction of accelerin by thrombin (WARE and SEEGERS 1948), and the consumption during prothrombin conversion (STEFANINI and CROSBY 1950, ALEXANDER, GOLDSTEIN and LANDWEHR 1951, and others) which continues some time after coagulation. The influences of these factors have not been studied separately in this work, regard being paid only to the mere disappearance of activity. By using defibrinated plasma instead of serum the influence of the two last mentioned factors was grossly excluded. Contrary to the findings of MURPHY and SEEGERS, accelerin was found to be present both in dog and human defibrinated plasma and serum, and in dog even in great amounts. The reason for this discrepancy is not clear.

By the experiments with acid precipitation it was shown that dog was the only species not showing a significant difference in the isoelectric point between proaccelerin and accelerin. Together with the small accelerin capacity found in this species, dog proaccelerin and accelerin seem to be less different than in the other species. However, as judged from the adsorption experiments, there is no doubt that they are different.

Accelerin was well adsorbed in all species, which conforms with the finding of LEWIS and WARE (1953) for human accelerin. In view of this the yield of accelerator after the method of GRAY, SCHAEFER and JENSEN (1956) must be limited, since they used barium sulphate adsorption after defibrination.

Summary and Conclusions.

1. Proaccelerin concentration in various species has been estimated as follows: Human 100, equine 300, bovine 500, and canine 900 per cent.

2. Comparison of accelerin capacity showed the following results: Human 22, equine 14, bovine 40, and canine 7.

3. Stability of proaccelerin was strongly influenced by pH. Between pH 6.5 and 7.0 stability was maximal, decreasing with increasing pH. The relation of stability to decalcification was confirmed.

4. Proaccelerin deterioration proceeded at least partly through activation to accelerin, which is more labile.

5. Proaccelerin and accelerin had different physico-chemical properties.

6. Species differences in physico-chemical properties were found for:

a) Stability. Bovine proaccelerin and accelerin were most stable, while the corresponding human materials were least stable.

b) Acid precipitation. Dog proaccelerin and accelerin had a definitely higher isoelectric point than the others tested. The isoelectric points under the conditions used were higher than previously thought.

c) Adsorbability. Only horse proaccelerin was easily adsorbed onto barium sulphate. Accelerin was well adsorbed in all species.

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Indirect Determination of Peripheral Blood Pressure in Mice during Light Anaesthesia.

By

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The methods described for the indirect measurement of blood pressure in mice involve compression of an artery and observation of the pressure at which circulation in the tail or legs begins on release of pressure (KUNSTMANN 1928, BEHRENS 1929, BONSMANN 1934, GRIFFITH 1934, MCMASTER 1941). The present method is a modification of the GRIFFITH-MCMASTER procedure (1934, 1941) by which recirculation was observed microscopically in the blood vessels of the foot.

Method.

The method involves compression of arteria plantaris pedis easily seen by the naked eye through the hairless skin on the medial aspect of the dorsal metacarpus. The animal was anaesthetized with ether until completely relaxed. The left hind foot was then swabed with ceder oil, placed in the glass viewing chamber (Fig. 1) with the toes protruding and the aperture was closed by placing a cork B under the foot with the wooden stick C. Air was pumped into the rubber balloon[1] in the viewing chamber by means of the bulb D until the circulation had stopped over two to three mm of arteria plantaris as observed with a KNISELY (1936) illumination device. The pressure was then released at a rate of 40 mm Hg per 10 sec. by opening the stop-cock E and allowing air to escape from the system. The pressure fall was arrested by blowing air into the syringe through F at that pressure which just allowed the pulsating blood column to refill arteria plantaris and the manometer was read at this point.

[1] Manufactured by Schönning and Arvé, Copenhagen. Appreciation is expressed to Mr. A. H. Rasmussen for its design.

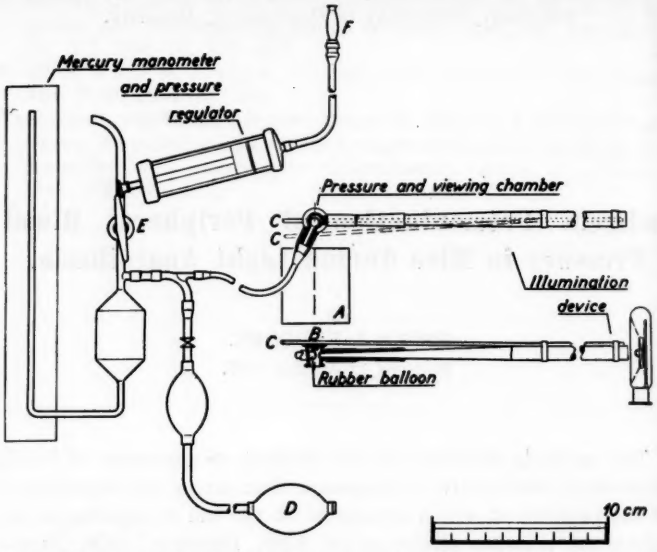

Fig. 1. Diagram of the blood pressure apparatus. Above: illumination device in the vertical plane, below: in the horizontal plane.
A: Glass-plate on which the mouse is placed.
B: Cork for closing the aperture under the foot of the animal.
C: Wooden stick for moving B.
D: Bulb to increase the pressure in the rubber balloon and the mercury manometer.
E: Stop-cock, in this position allowing a pressure fall in the system of 40 mm Hg per 10 sec.
F: Mouth-piece.

In practice two blood pressure measurements were taken with the ether mask still in place. The mask was then removed and the blood pressure measured every 15 sec. until the animal withdrew its leg, usually after two or three determinations. The average of the last two measurements before foot withdrawal was selected to indicate the blood pressure.

Results.

Normal Arterial Blood Pressure.

The blood pressures of 40 normal white male mice (AKA strain, weight 18—27 g) was 60—120 mm Hg. The average blood pressure was 90 mm Hg, mean variation 13.1 mm Hg, s. d. 15.4 mm Hg. The day to day variation in any one mouse as judged from four mice measured daily for 12—26 days lay within this

Table 1.

Four blood pressures of ten normal mice at intervals of one hour.

Mouse 1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
87	93	96	102	64	101	91	97	105	111
87	92	85	100	109	104	92	101	112	117
76	95	86	101	110	102	93	102	69	100
88	81	97	102	83	100	92	101	93	95

range. Hour to hour variations are shown in Table 1 as measured four times for each of ten mice at one hour intervals. The mean variation in one day was 5.9 mm Hg, s. d. 10.4 mm Hg.

In ten mice *ether anaesthesia* of such depth that the time from removal of the mask to withdrawal of the foot was five minutes or more was associated with blood pressures exceeding the highest values found by the standard procedures. The blood pressure values fell within the normal range when the period from mask removal to foot withdrawal was less than five minutes. By the standard procedure it was less than two minutes.

Discussion.

The blood pressures found with the method described here correspond to the normal values reported by BEHRENS (1929) and MCMASTER (1941). MCMASTER (1941) found agreement between simultaneously performed direct and indirect blood pressure measurements. The blood pressure values of BONSMANN (1934) and KUNSTMANN (1928) were slightly lower possibly because their methods depended on capillary recirculation as criterion for the blood pressure.

The advantage of the present method was that attention could be concentrated on observation of the artery. In addition the method was easy and quick and reproducible values were found. Finally, it could be used on very lightly anaesthetized animals.

Summary.

A method is described for measuring blood pressure in mice during light ether anaesthesia. The method involved compression of the arteria plantaris pedis with air in a glass chamber under strong illumination. During release of pressure, the pressure was arrested when blood just refilled the artery. Values at this point varied between 60—120 mm Hg in normal mice.

This work was supported by grants from the King Christian the Xth Foundation and Miss P. A. BRANDT's Bequest.

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A Comparative Study of Prostaglandin from Human Seminal Fluid and from Prostate Gland of Sheep.

By

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In extracts as well as in secretions from human prostate gland and seminal vesicle EULER in 1934 and 1936 demonstrated the occurrence of an active principle which had a depressor effect upon the rabbit's blood pressure and stimulated many smooth muscle organs like the intestine and uterus of the rabbit. This principle, tentatively named prostaglandin (EULER 1935), could also be demonstrated in the accessory glands of sheep (EULER 1934, 1939), while prostate glands and seminal vesicles from other animals investigated showed no such activity (EULER 1934). In the sheep other organs also seemed to contain small amounts of the same principle, but the quantity found per unit weight of tissue was always less than one per cent of that seen in the prostate gland (EULER and HAMMARSTRÖM 1937).

The chemical and biological properties of the active principle were studied by EULER (1936, 1939) and a more complete chemical investigation of prostaglandin extracted from prostate glands of sheep was reported by BERGSTRÖM (1949). According to these studies prostaglandin is a fatty acid, probably unsaturated, and contains no nitrogen. If injected intravenously, it lowers the blood pressure in the cat and the rabbit. It also causes contraction of many smooth muscle organs. The physiological function of the substance is unknown, although its presence in the accessory genital gland secretions suggests a role in the reproductive function.

EULER (1934—1939) found that the biological effects of the two different prostaglandin preparations on smooth muscle organs were very similar. ASPLUND (1947) found that human seminal fluid and prostaglandin prepared from prostatic glands of sheep had the same type of stimulating effect also on the rabbit's uterus in vivo. Direct comparative studies to prove the identity of the two substances are, however, still lacking. The present investigation in which some chemical and physiological properties of prostaglandin obtained from the two different sources are compared was carried out to elucidate further whether the two prostaglandin preparations are identical or not.

Methods.

Extraction of prostaglandin.

1) *Collected human semen* was kept in a refrigerator at — 20° C. When used, it was warmed at room temperature and centrifuged. The supernatant fluid was made acid (pH 3—5) with hydrochloric acid and 4—5 volumes of acetone were added. The solution was heated to boiling and thereafter filtered. The acetone was evaporated under reduced pressure and the residue made weakly alkaline with NaOH under slight warming. After extraction of the unsaponified material with 10 volumes of ether, repeated twice, the water phase was made acid and again extracted twice with 10 volumes of ether. The ether was evaporated, the residue dissolved in a small amount of 0.01 n NaOH, pH adjusted to about 7.5 and phosphate buffer added to the desired volume.

2) *The prostate glands* were freed from surrounding tissue, ground and mixed with 2 volumes of acid alcohol (1 ml 3 N HCl per 200 ml 95 % ethanol). After rapid heating to boiling the solution was filtered and the filtrate placed in a refrigerator (0° C) overnight. The solution was again filtered and the clear filtrate evaporated under reduced pressure until 1 ml solution contained the equivalent of 20—30 gram tissue. The pH was adjusted by sodium hydroxide to about 7.5 under slight warming. The unsaponified matter was extracted with ether and after acidification, the fatty acid material was extracted with ether, similar to the procedure used for seminal fluid. Some of the preparations were further purified by separating the saturated and unsaturated fatty acids using the lead-acetate method described by HILDITCH (1949).

Thermostability. Prostaglandin samples containing about 20 units per ml were boiled in a water bath for 20 minutes. The pH ranged between 0.5 and 12, determined by pH-meter. Hydrochloric acid and potassium hydroxide were used respectively to obtain the desired pH. After boiling the pH was adjusted to 7 and the activity was tested on isolated rabbit jejunum.

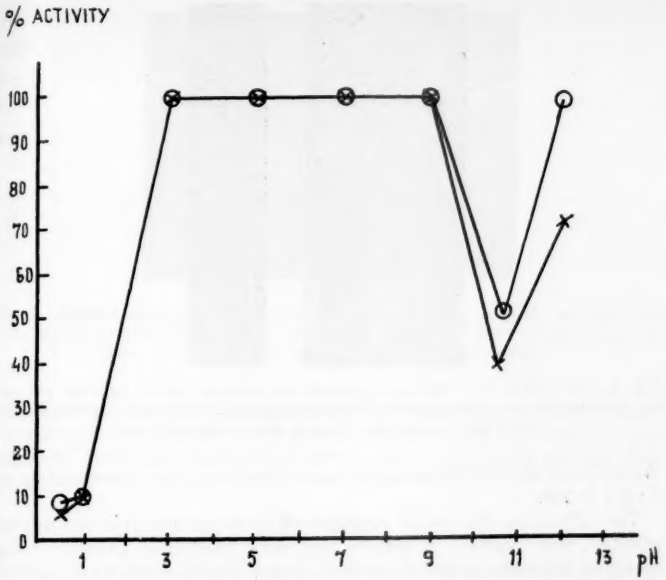

Fig. 1. Per cent prostaglandin activity left after 20 minutes' boiling at different pH. X = prostaglandin from human seminal fluid. O = prostaglandin from prostate gland of sheep.

Paper chromatography. The paper used was Whatman No. 1. The chromatograms were developed 12 hours in an atmosphere of nitrogen by the ascending method described by BERRY, SUTTON, CAIN and BERRY (1951). The two substances were run both separately and mixed. The activity was located by cutting the paper strips in pieces of 1 cm of length and immersing each piece in an isolated organ bath. If present, the active principle was rapidly eluted and gave rise to a contraction of the isolated rabbit jejunum. The solvents used were:methylethylketone/diethylamine/water (60: 3 : 20); ethyl acetate/acetic acid/water (3 : 1 : 1); methylglycol/conc. NH_4OH/water (80 : 5 : 15).

Biological assays were done on the isolated rabbit jejunum, rat jejunum, guinea-pig ileum and guinea-pig uterus. The guinea-pig organs were suspended in a 3 ml bath, the others in a 15 ml bath. Tyrode solution at 38° C was used and for the guinea-pig organs the solutions were aerated with oxygen, and for other organs with 6.5 % carbon dioxide in oxygen. Biological tests were performed both with and without atropine and an antihistamine agent[1] in the Tyrode solution.

[1] Antasten ®, kindly supplied by Ciba, or Thiergan ®, kindly supplied by Astra.

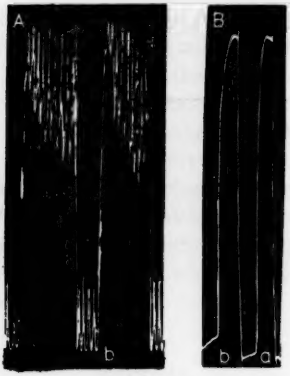

Fig. 2. The effect of 1 unit prostaglandin on isolated rabbit jejunum (A) and rat jejunum (B). a = prostaglandin from human seminal fluid, b = prostaglandin from prostate gland of sheep. Bath volume 15 ml.

When used, the two antagonists were added to give concentration of $1 : 2.5 \times 10^6$.

The effect on the blood pressure of intravenously injected prostaglandin was studied in rabbits and cats, the blood pressure being recorded from the common carotid artery. The samples were injected into the femoral vein. Tests were performed with and without atropine and antihistamine.

During the purification procedures biological assays were made in isolated rabbits jejunum or duodenum.

As standard an extract from prostate gland of the sheep was used, its strength given in units as defined by EULER (1939).

The preparation obtained from the human seminal fluid as described above shows an activity equal to 8—10 units per mg free acid.

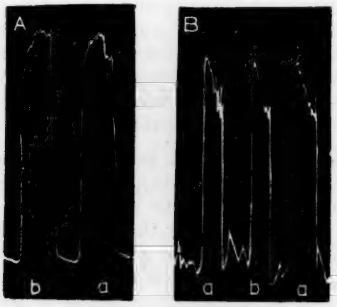

Fig. 3. The effect of 2 units prostaglandin on isolated uterus (A) and ileum (B) from guinea-pig. a) and b) as in figure 2. Bath volume 3 ml.

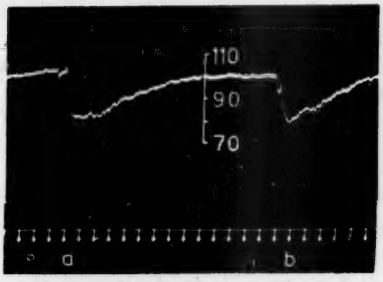

Fig. 4. Rabbit blood pressure. At a) intravenous injection of 1 unit prostaglandin from human seminal fluid, at b) 1 unit of prostaglandin from prostate gland of sheep. Atropin 2 mg per kilogram. Time 1 minute.

The prostaglandin preparation obtained from prostate glands of sheep contained 3—5 units per mg free acid and after the separation of the saturated from the unsaturated fatty acids 8—10 units per mg free acid.

Results.

Thermostability. Figure 1 shows that the active principle is stable between pH 3 and 9. The partial inactivation at pH 10.5 and good thermostability at pH 12 have been regularly found.

Paper chromatography gave the same Rf-values for the two preparations. These were 0.40—0.50 in methylethylketone/diethylamine/water, and 0.8 in ethyl acetate/acetic acid/water.

Biological assay. When the two preparations were tested on the isolated organs the qualitative effects were always the same and compared with the standard, the ratio observed was also identical (figs. 2 and 3).

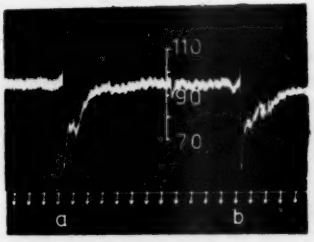

Fig. 5. Cat blood pressure. The effect of 12 units prostaglandin injected intravenously. a) and b) as in figure 4. Atropin 2 mg per kilogram. Time 1 minute.

When injected intravenously the two prostaglandin preparations gave a rapid lowering of the blood pressure. The type and degree of the depressor effect were similar for the two preparations (figs. 4 and 5).

Summary.

1. Prostaglandin extracted from human seminal fluid and from prostate gland of sheep have been compared chemically and biologically.

2. It has not been possible to distinguish the two substances by paper chromatography, thermostability or biological tests, including rabbit jejunum, rat jejunum, guinea-pig ileum, guinea-pig uterus, rabbit blood pressure and cat blood pressure.

This investigation has been supported by grants from the Therese and Johan Anderssons Minne, the Lars Hiertas Minne and Karolinska Institutet, Stockholm.

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Pyrrolidone Carboxylic Acid in Enzymatic Casein Hydrolysates.

By

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Received 21 January 1957.

In microbiologic analysis of a dialyzed enzymatic casein hydrolysate with *Lactobacillus arabinosus*, it was found that on direct determination of the glutamic acid content, it was possible to demonstrate only 37—38 per cent of the quantity found after complete hydrolysis with hydrochloric acid (WRETLIND 1952). It was therefore concluded that the glutamic acid in the preparation was present partly in a "bound" form. Determination of the "bound" glutamic acid was relatively easy, since its quantity could be obtained as the difference between the glutamic acid content before and after complete hydrolysis.

An account is given in the present paper of the demonstration, isolation and analysis of this "bound" glutamic acid, and of how it was found to consist largely of pyrrolidone carboxylic acid, formed during digestion and heat treatment of the enzymatic casein hydrolysate.

Experimental.

The investigations were made on a dialyzed enzymatic casein hydrolysate, aminosol[1], described and analyzed earlier by WRETLIND (1952). Use was also made of preparations corresponding to aminosol, except that they were taken at various stages after enzymatic digestion with trypsin and erepsin.

Microbiologic determination of glutamic acid was performed with *Lactobacillus arabinosus 17-5*, according to HENDERSON and SNELL (1948), as described earlier (WRETLIND 1952).

[1] Aminosol is manufactured by Vitrum, Stockholm.

The first attempts to demonstrate the "bound" glutamic acid were made with the chromatographic technique. A starch column was used, 50 cm high and 9 cm in diameter, in equilibrium with a mixture of one part *n*-butanol, 2 parts *n*-propanol and 1 part 0.01 *N* hydrochloric acid (ÅQVIST 1951). 2.22 g of aminosol powder were dissolved in 70 ml of 0.01 *N* hydrochloric acid, with the addition of 140 ml of *n*-propanol and 70 ml of *n*-butanol. The mixture was transferred to the starch column, which was then eluted with the aforementioned butanol-propanol-0.01 *N* hydrochloric acid mixture. It was shown by preliminary experiments that, with this technique, the "bound" glutamic acid was eluted with a volume of less than 3 litres. Elution was therefore terminated in these experiments when this volume was obtained. Close fractions were taken from the eluate.

For microbiologic analysis of the different fractions, an exact volume of each fraction was evaporated to dryness *in vacuo*, and then dissolved with distilled water to the original volume. 10-ml portions of this solution were put in test tubes together with equal volumes of concentrated hydrochloric acid, and the tubes placed in a boiling water bath. After 24 to 36 hours the fluid had evaporated; the residue was dissolved in distilled water to the original volume and filtered. The solutions before and after acid hydrolysis were adjusted to pH 6.0 before the microbiologic analysis. In those cases in which the glutamic acid concentration was found to be too high, the solutions were diluted. The results of microbiologic determination of glutamic acid before and after hydrolysis of the different fractions are shown in Fig. 1.

For colorimetric analysis with a ninhydrin method according to MOORE and STEIN (1948), 0.1 ml of each fraction was taken. A further 0.1 ml was pipetted off into small vials (7.5 × 0.5 cm), evaporated to dryness in a vacuum exsiccator, and 0.2 ml of 6 *N* hydrochloric acid added. The vials were sealed by melting and placed in a boiling water bath for 24 hours. At the end of hydrolysis, the vials were opened and once more evaporated to dryness *in vacuo* over solid sodium hydroxide. The samples were analyzed with the aforementioned ninhydrin method. The results of colorimetry before and after hydrolysis are recorded in Fig. 1. Paper chromatography was performed on samples from different fractions hydrolyzed with hydrochloric acid, using *sec*-butanol, acetic acid and water (40 : 10 : 10) as the solvent. The paper used was Whatman no. 1, and the spots were developed with ninhydrin.

Isolation of pyrrolidone carboxylic acid was performed by allowing an aminosol solution to run through a column of the cation exchanger Dowex 50 in H^+ form. All the free amino acids are bound in this way, and only any acid peptides present, as well as the acids, including pyrrolidone carboxylic acid, can be eluted with water. The pyrrolidone carboxylic acid was extracted from the water eluate with ethyl acetate, and crystallized from acetone.

The detailed procedure for isolation of pyrrolidone carboxylic acid is as follows. Seventy g of aminosol powder are suspended with stirring at room temperature for 1 hour in 200 ml of distilled water. The solution is placed in the refrigerator overnight, after which the precipitate and

undissolved components are filtered off. The filtrate is allowed to pass through a column of Dowex 50—X 12 (200—400 mesh). The diameter of the column is 40 mm, and the volume of Dowex 50 is 300 ml. After the amino acid filtrate has been added, an additional 200 ml of distilled water is run through the column. The collected volumes of fluid that have run through the column are freeze-dried to a paste, which is dissolved with 15 ml of distilled water, after which 300 ml of acetone are added with stirring. The sticky precipitate thus formed is separated off; the acetone is evaporated on a water bath and distilled water added to a volume of 30 ml. The aqueous solution is then extracted for 24 hours with ethyl acetate in a continuous fluid-fluid extraction apparatus. The pyrrolidone carboxylic acid is then transferred to the flask with ethyl acetate. After evaporation of the ethyl acetate on a water bath, the viscid, damp residue is dissolved in 30 ml of boiling acetone. The acetone solution is filtered hot and placed in the refrigerator, after which the pyrrolidone carboxylic acid that has crystallized out is separated by filtration, and washed with 2 portions of 5 ml of acetone and 10 ml of ether. The mother lye is evaporated to a volume of about 10 ml and kept in the refrigerator overnight, after which further crystalline masses of pyrrolidone carboxylic acid are obtained. They are filtered off and washed as stated earlier. The yield of pyrrolidone carboxylic acid varies from 0.8—1.9 g. Melting point 159—161° C. Elementary analysis: N found 10.7—10.8 per cent, calculated 10.85 per cent.

The isolation procedure was checked with pure L-pyrrolidone carboxylic acid. The experiment was then performed with a solution containing 5.0 g in 200 ml of water, and it was found that after treatment with Dowex 50, 4.9—5.0 g were recovered after freeze-drying. After ethyl acetate fractionation for 24 hours, 0.4—0.9 g remained in the water phase and 4.6—4.1 g had been converted via ethyl acetate. Of the quantity converted, 3.3—3.5 g were obtained in crystalline form after the aforementioned crystallization procedure from acetone. When ethyl acetate extraction was allowed to proceed for 48 hours, 98 per cent of pyrrolidone carboxylic acid was converted. It was, however, found that this lengthy extraction could not be used in the isolation of pyrrolidone carboxylic acid from aminosol, since a number of dark-coloured substances were then transferred, which made crystallization of the acid more difficult.

Paper chromatographic demonstration of pyrrolidone carboxylic acid. The pyrrolidone carboxylic acid was demonstrated by descending paper chromatography, using *sec*-butanol-acetic acid-water (40 : 10 : 10) and Whatman no. 1 paper. When chromatography had proceeded for 14—24 hours at room temperature, the solvent was evaporated by keeping in an oven at 130° C for $^1/_2$—1 hour. The spot with an acid reaction due to pyrrolidone carboxylic acid was demonstrated by spraying the paper with an indicator solution containing 0.1 per cent bromophenol blue dissolved in 50 per cent ethanol. Pyrrolidone carboxylic acid and other acid substances then appear as yellow spots against a blue background. A suitable concentration of pyrrolidone

carboxylic acid lies between 0.4 and 1.2 per cent, and the quantity applied was 0.01 ml. In these cases, the R_f value for pyrrolidone carboxylic acid was 0.61.

An example of a chromatogram of pyrrolidone carboxylic acid is given in Fig. 2. In this figure are also shown chromatograms of aminosol after passage through Dowex 50, the fractions obtained after extraction with ethyl acetate, and the crystalline pyrrolidone carboxylic acid produced from aminosol with the method described above.

Ion exchange separation with Dowex 2 for analysis and identification of pyrrolidone carboxylic acid. 760 ml of aminosol (3.3 per cent) were added to a short column of Dowex 50 in H^+ form (9.5 × 8.0 cm), washed with water, and then eluted with water until ninhydrin-positive substance could no longer be demonstrated in hydrolyzed samples. This material of acid peptides and other acid substances is freeze-dried to dryness and dissolved in 25 ml of 0.5 *M* acetic acid, after which it is put into a 60 × 5 cm column of Dowex 2—X 8 in acetate form, 200 mesh (HIRS, MOORE and STEIN 1954). The column is then eluted in steps with 0.5, 2.0 and 5.0 *M* acetic acid (Fig. 3). Ninhydrin colorimetry on hydrochloric-acid-hydrolyzed samples from the different fractions showed a number of ninhydrin-positive substances, in which pyrrolidone carboxylic acid appeared as a large peak after elution with 5 *M* acetic acid. Closer chromatographic analysis showed that, after hydrolysis, it was entirely free from other amino acids than glutamic acid. On evaporation of the relevant fractions, the substance crystallized directly in the flask. It was demonstrated by control experiments with pure pyrrolidone carboxylic acid that this appeared at the identical place on the chromatogram as the aforementioned substance isolated from aminosol.

Quantitative analysis of aminosol was made both on the solution for parenteral use and on the powder for oral administration. The fractions of 5 *M* acetic acid containing pyrrolidone carboxylic acid were then collected, evaporated to dryness *in vacuo* and hydrolyzed with hydrochloric acid. The hydrolysate was evaporated to dryness and transferred quantitatively with water to 30 × 0.6 cm columns of Dowex 50 in H^+ form. Glutamic acid was then eluted with *N* HCl. The total nitrogen content of these fractions was determined according to Kjeldahl, and on the basis of this value the pyrrolidone carboxylic acid N was calculated as a percentage of the quantity of nitrogen in the aminosol analyzed.

Corresponding analyses were made on an enzymatic casein hydrolysate taken after hydrolysis for varying periods. This was prepared by dissolving 50 g of casein in 1,000 ml of distilled water, and adding 1 *N* NaOH to obtain a pH of 7.6. After addition of 3.0 g of acetone-dried pancreas (WRETLIND 1952) the mixture was allowed to stand at 37° C, with continuous control of the pH, the pH being adjusted to 7.6 with 1 *N* NaOH. After 13 days, 4.0 g of bovine intestinal mucosa was added. The samples taken were analyzed for their content of pyrrolidone carboxylic acid, total nitrogen and amino nitrogen. The results are recorded in Fig. 4.

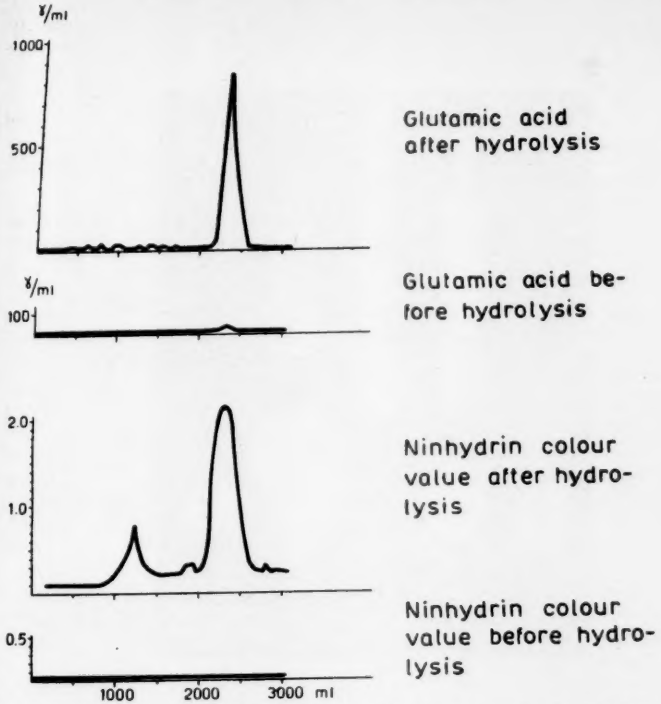

Fig. 1. Analysis of glutamic acid by microbiologic determination and with ninhydrin, before and after hydrolysis of the different fractions obtained after chromatography of aminosol. Starch was used as the column for chromatography with *n*-butanol-*n*-propanol-0.01 *N* hydrochloric acid (1 : 2 : 1) as solvent. The upper two curves show the quantity of glutamic acid in mg/ml determined microbiologically, after and before hydrolysis, respectively. The lower two curves show the extinction values for the ninhydrin-positive substances after and before hydrolysis, respectively. — The quantity of eluate is given on the abscissa.

Results and Discussion.

Fig. 1 shows that, with the microbiologic technique, it was easy to demonstrate the "bound" glutamic acid as well-defined fractions on starch chromatography. That there was no contamination by free glutamic acid was demonstrated by the fact that, before hydrolysis, practically no glutamic acid was obtained with the microbiologic technique. The simultaneous ninhydrin reaction performed on the different fractions showed that no ninhydrin-positive substances were present before hydrolysis (Fig. 1). In

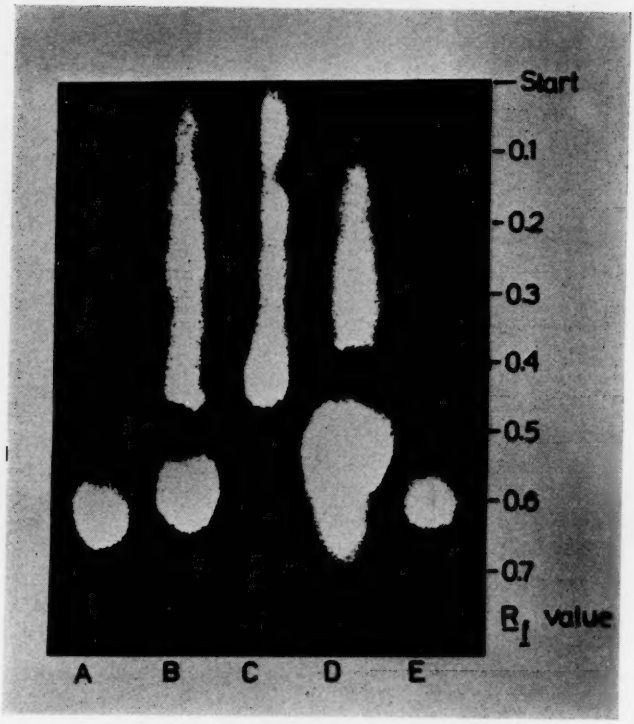

Fig. 2. Chromatogram of pure pyrrolidone carboxylic acid and of samples from different phases during isolation of pyrrolidone carboxylic acid from aminosol. Whatman no. 1 paper was used and *sec*-butanol-acetic acid-water (40 : 10 : 10) as solvent. Descending chromatography was performed at room temperature and continued for about 14 hours. After drying, 0.1 % bromophenol blue in 50 % ethanol was used for development. A. 0.01 ml 1 % L-pyrrolidone carboxylic acid. B. 0.01 ml aminosol solution (70 g aminosol dissolved in 200 ml H_2O), which had passed through Dowex 50—X 12 in H+ form. C. 0.01 ml of B after extraction with ethyl acetate. D. 0.01 ml of the fraction from B extracted and transferred with ethyl acetate. E. 0.01 ml of 1 % solution of crystalline pyrrolidone carboxylic acid prepared from aminosol with the isolation procedure described in the text.

the samples hydrolyzed with hydrochloric acid, distinct colour reactions were observed, thus showing the presence of ninhydrin-positive substances, *i. e.*, in this case amino acids. Paper chromatography of the fractions hydrolyzed with hydrochloric acid showed the presence of numerous amino acids in most samples. The

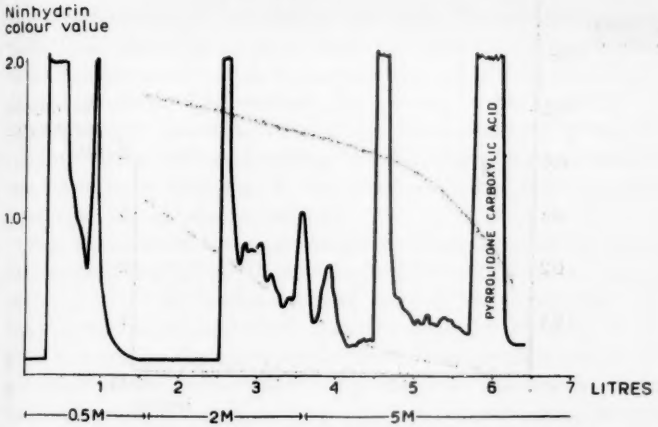

Fig. 3. Chromatographic analysis on Dowex 2 of substances from aminosol which had passed through Dowex 50 with water. Dowex 2—X 8 was used in acetate form, and elution performed with acetic acid in the rising concentration shown below the abscissa. The different fractions were analyzed with the ninhydrin reaction after hydrolysis with hydrochloric acid. The extinction values then obtained are shown on the ordinate. The quantity of eluate is given on the abscissa.

samples from the fractions in the 2,000—2,600 ml region were, however, dominated by glutamic acid, even if traces of other amino acids occurred. Nor could the material be separated with absolute purity on starch columns with other partition chromatographic methods. This was, however, successful with the use of the ion exchange technique with Dowex 2.

Certain conclusions regarding the properties of the "bound" glutamic acid could be drawn from this first chromatographic analysis and from similar ones. Thus, it must have been a low-molecular substance, which was ninhydrin-negative and which contained almost exclusively glutamic acid as ninhydrin-positive substance after acid hydrolysis. Furthermore, it was found to pass through the cation exchanger Dowex 50 in H^+ form, which showed that the substance was an acid. It was concluded from these observations that it might be pyrrolidone carboxylic acid. In order to perform control determinations, pyrrolidone carboxylic acid was prepared from L-glutamic acid by heating in aqueous solution to 135° C according to DOLINEK (1927/28). It was then found that the pyrrolidone carboxylic acid was ninhydrin-negative, and did not produce growth in *Lactobacillus arabinosus.*

Fig. 4. Amino nitrogen and pyrrolidone carboxylic acid content during enzymatic digestion of casein. Digestion of a 5 % casein solution was performed with pancreatic and intestinal hormones. The ordinate shows both the ratio of amino nitrogen to total nitrogen, and the pyrrolidone carboxylic acid content as a percentage of the total nitrogen content. The abscissa gives the time of digestion in hours on a logarithmic scale.

Moreover, chromatographic analysis showed the pyrrolidone carboxylic acid to behave in exactly the same way as the "bound" glutamic acid in aminosol.

In order to prove that it was, in fact, pyrrolidone carboxylic acid, the isolation procedure described was worked out. All the amino acids and peptides with basic groups are removed first. From the fractions of acids and acid peptide compounds thus obtained, a number of substances are precipitated by means of 95 per cent acetone, in which pyrrolidone carboxylic acid is readily soluble. After extraction with ethyl acetate, which has been used by several workers (PUCHER and VICKERY 1940, RATNER 1944, KÖGL, BARENDREGT and KLEIN 1948), crystalline pyrrolidone carboxylic acid was obtained from acetone. This can be regarded as proof that pyrrolidone carboxylic acid is contained in these casein hydrolysates. The relatively good yield in experiments with the pure substance shows that the method is well suited for isolation of the acid in question.

A simple method for demonstrating pyrrolidone carboxylic acid by paper chromatography with the use of indicators facili-

tated elaboration of the isolation procedure. This chromatographic technique could, however, be used only after removal of all such substances as amino acids by passage through a column of Dowex 50 in H^+ form. The method also proved to be applicable for approximative quantitative analysis. The behaviour of the pyrrolidone carboxylic acid during the different phases of isolation can be inferred from Fig. 2, and gives additional evidence of the purity of the substance isolated.

Fig. 3 shows how the pyrrolidone carboxylic acid is isolated in chromatography on Dowex 2—X 8 in acetate form. Quantitative analysis with this method showed the pyrrolidone carboxylic acid nitrogen content to amount to 7.7 per cent of the total nitrogen in the aminosol for oral administration, and 4—4.1 per cent in the aminosol for parenteral use. The change in the ratio of amino nitrogen to total nitrogen in relation to the pyrrolidone carboxylic acid content is seen in Fig. 4. It can be inferred that the quantity of pyrrolidone carboxylic acid increases considerably more slowly than the degree of proteolytic hydrolysis. The content increases successively until the end of digestion (34 days) when it is 3.1 per cent. Subsequent heat treatment raises the values further until they reach those just mentioned for the final preparations.

The problem of pyrrolidone carboxylic acid formation in protein hydrolysates has been dealt with by MELVILLE (1935) among others. He showed that glutamine is formed in trypsin digestion of casein, and by using indirect, titrimetric methods he was able to calculate and to demonstrate that glutamine is probably converted into pyrrolidone carboxylic acid. These experiments were made both with pure glutamine and with protein hydrolysates at pH 7.05 and 37° C. It was established by CHIBNALL and WESTALL (1932) that glutamine is converted into pyrrolidone carboxylic acid even by heating to 100° C and at pH 6—7. Consequently, it seems justified to assume that the pyrrolidone carboxylic acid demonstrated in the present investigation was formed from the glutamine obtained on enzymatic hydrolysis. The quantity of the acid in question is dependent on the length of digestion and on the subsequent heat treatment and sterilization by heat.

The body's ability to utilize glutamic *acid* when it occurs as pyrrolidone carboxylic acid seems to be good when the preparation is given orally. Experiments made by SCHLÜTZ (1953) and

by PEDERSEN and LEWIS (1944) have shown that on oral administration of L-pyrrolidone carboxylic acid, the major part is hydrolyzed in the gastrointestinal tract. Consequently, this implies that glutamic acid in the form of pyrrolidone carboxylic acid is utilized on oral administration. — With respect to subcutaneous administration, experiments of SCHLÜTZ (1953) in the rabbit indicate that the greater part of the pyrrolidone carboxylic acid injected is excreted unchanged in the urine. The conditions in man are not yet elucidated. According to HOWES and PUTTER (1954) an advantage of glutamic acid in the form of pyrrolidone carboxylic acid is that the side-effects produced by glutamic acid are then lacking.

It is apparent from the analyses of the aminosol preparations that the quantities of pyrrolidone carboxylic acid found are not entirely in agreement with the figures given earlier for the content of "bound" glutamic acid (WRETLIND 1952), which cannot be determined microbiologically before hydrolysis. This implies that these preparations contain other impurities, probably peptides, with glutamic acid in such a form that it cannot be utilized by the microorganisms.

Summary.

An investigation has been made of the "bound" glutamic acid present in an enzymatic casein hydrolysate, aminosol, and which is determined as the difference between the values obtained microbiologically before and after hydrolysis of the preparation with hydrochloric acid. Part of this "bound" glutamic acid is found to consist of pyrrolidone carboxylic acid.

Pyrrolidone carboxylic acid is isolated and analyzed in different casein hydrolysates, in which it is probably formed from glutamine.

The pyrrolidone carboxylic acid content of the enzymatic casein hydrolysates investigated (aminosol) lies between 4.0 and 7.7 per cent.

The quantity of pyrrolidone carboxylic acid in the enzymatic casein hydrolysates rises continuously during the 34-day digestion period, and is probably formed from the glutamine freed during enzymatic digestion.

In addition to pyrrolidone carboxylic acid, "bound" glutamic acid is also present in other forms in the enzymatic casein hydrolysates.

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The Flicker Electroretinogram of the Human Eye.

By

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Whereas the electroretinogram (ERG) on stimulation with single flashes chiefly reflects rod function, the flicker ERG with a stimulus light over 10 lux provides an objective test of the cones (DODT 1951, BORNSCHEIN and SCHUBERT 1953, DODT and WADENSTEN 1954). Thus, according to the investigations of DODT, photopic potentials are found in the flicker ERG that are characterized by the presence of both on- and off-effects.

With the apparatus and method used at our clinic, it has now been possible to analyze the photopic flicker ERG in some detail. A brief account of some general observations is given below.

Method.

The placing of the electrodes was, on the whole, in accordance with KARPE's method (1945). However, in order to obtain even illumination of the greatest possible area of the retina, use was made of a matt contact glass (a plastic glass covered with a thin layer of fabric). The retinal potentials were led off through a condenser-coupled amplifier with a time constant of 70 msec to one beam of a double-beam oscillograph, its other beam being used for recording, by means of a photocell, the stimulus period. By synchronizing the sweep and the photocell, the ERG could be photographed at a constant flicker rate in the form of about 10 superimposed traces, on a stationary film. Artefacts could easily be ruled out by means of this method of recording.

A Philips Tungsten Ribbon lamp (Type W 1) with a constant temperature of 2,800° K was used as the source of light. The light beam passed through a condenser, and thereafter through a 2 mm wide slit, which was opened and shut through a relay.

The experimental subjects consisted of 5 persons, between 20 and 30 years of age, with clinically normal eyes.

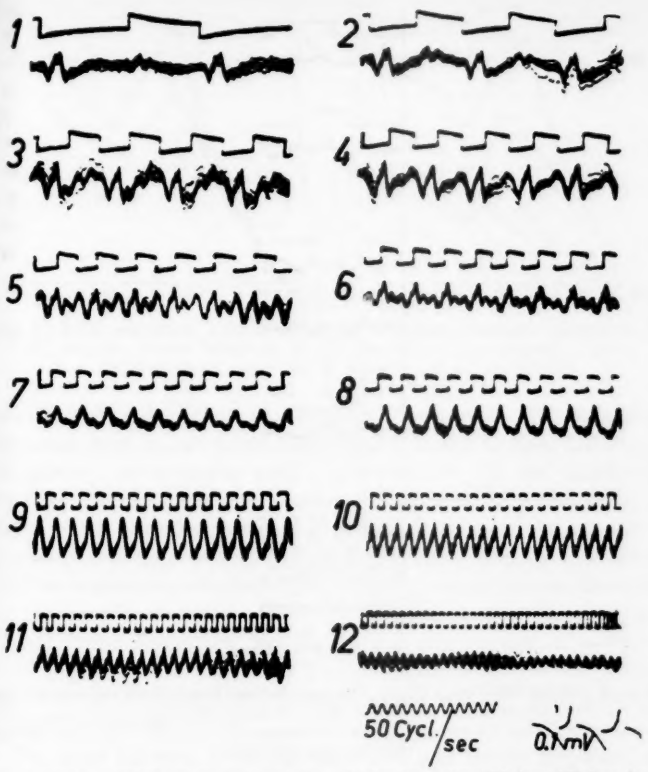

Fig. 1. Flicker ERG. Matt contact glass. Light intensity 250 lux. Flicker frequency increased from records 1—12 (2.5, 5, 7.5, 10, 12.5, 16.5, 20, 22, 30, 36, 45, and 67/sec., respectively). Light: darkness = 1 : 1. In all records, the uppermost line is the light signal (downward = on, upward = off); below the ERG. Below and to the right: time marking and calibration. By synchronizing the sweep and photocell, 10 superimposed records have been photographed at each flicker frequency (this also applies to Figs. 2 and 4).

Results.

The normal flicker ERG at a light intensity of 250 lux is shown in Fig. 1 below a stimulus signal. The flicker frequency is constant in each individual record, but increases from no. 1 to no. 12. A striking feature is the transition from a polyphasic to a diphasic type of curve during increasing flicker frequency. Whereas in record 1 there is one *a*-wave, a three-peaked *b*-wave and a double

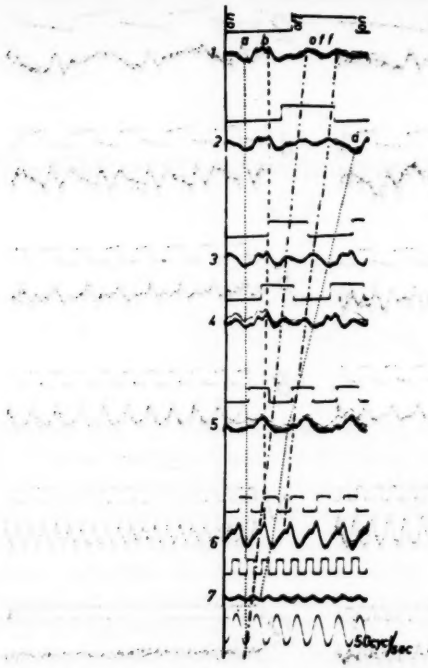

Fig. 2. Flicker ERG as in Fig. 1. The records have been placed below each other so that the perpendicular distance is proportional to the logarithmic value of the increase in flicker frequency. (Flicker frequency: 8.5, 9.5, 12.5, 15.5, 25, 40 and 67/sec., respectively.)

off-effect, none of these details are present in no. 12. Furthermore, with rising flicker frequency amplitude of potential changes as a result of interference between the different phases. The way in which this interference picture arises can be studied in greater detail in Figs. 2 and 3.

In Fig. 2, all the curves start with onset of light. They have been placed below each other, but at a varying distance, so that the perpendicular distance between two curves is proportional to the logarithmic value of the increase in flicker frequency. This makes it easier to study the different ERG phases, which are joined in the figure by vertical lines.

All the aforementioned phases are present in record 1. When the flicker frequency is increased, the 2nd positive off-effect in

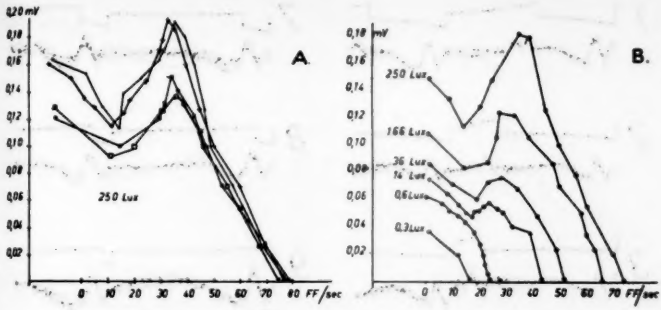

Fig. 3. ERG amplitude with rising flicker frequency. Ordinate: amplitude in mV. Abscissa: flicker frequency. A. Results with 4 experimental subjects. B Different light intensities in experiments on one subject

one record approaches the negative *a*-wave in the following response (nos. 2—4). Since these waves, owing to their difference in latency, subsequently tend to coincide (no. 5), the amplitude at first decreases and the curve recorded has a simplified appearance. With a further increase in flicker frequency the off-effect becomes superimposed on the positive *b*-wave and by this interference maximal potentials arise. Finally, a continuous decrease in the amplitude takes place, until fusion occurs. Close to the flicker fusion frequency, the flicker response consists of *a*-waves only together with the first off-effect, whereas the other phases (the *b*-wave and the 2nd off-effect), owing to their long latency, cannot be traced.

The ratio between potential amplitude and flicker frequency is shown graphically in Fig. 3. If the greatest amplitudes in each record are measured at the different frequencies given in Fig. 1, a curve is obtained with a minimum of potential at 10—15 flashes/sec., a maximum at 30—40/sec., and a practically linear decrease in amplitude from this frequency until fusion occurs (Fig. 3 A).

The values for the minimum and maximum in this curve are dependent on the intensity of the stimulus light (Fig. 3 B). With a scotopic flicker response (intensity below 10 lux), a considerably simpler curve is obtained. This is because, under such conditions, no off-effect appears and consequently no interference between it and the *b*-potential. This explains the sudden increase in amplitude in Fig. 2 and is in good agreement with the results of DODT and WIRTH (1953), who were able to reduce the

Fig. 4. Flicker ERG with varying light-dark ratio. Records 1—6: decreasing dark interval; 7—11: decreasing light interval. — All records start with "on".

influence of the off-effect by changing the ratio of the light to dark periods in the flicker.

The subjective sensations are a further indication that the increase in the amplitude of the potentials is due to algebraical interference, since no increased brightness of light is perceived in the course of the experiment.

Fig. 4 shows the results of an experimental series in which the interval of darkness was decreased successively. It is seen in record 1—6 how the off-effect submerges into the following *a*-wave.

In records 7—11 in Fig. 4, the duration of the light stimulus has been successively decreased. In order to ensure constant light adaptation under these conditions, each record was preceded by a 3-minute period of constant flicker 1 : 1. Since, despite this

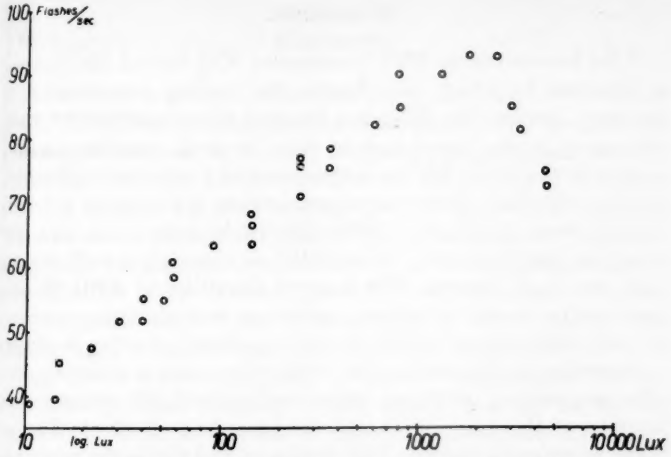

Fig. 5. Flicker fusion frequency in relation to light stimulus. Ordinate: Flicker frequency. Abscissa: log light stimulus (in lux). — Each circle denotes the mean value of 10 determinations in three different experimental subjects.

fact, the *b*-waves in records 9 and 10 are distinctly larger than those in nos. 7 and 8, it must be assumed that this enlargement is due to a superimposition of off-effects. In other words, the off-effect can, in a light-adapted eye, be demonstrated even with brief light stimulation, a fact that WIRTH and MÜLLER-LIMMROTH (1955) were able to show by the use of double stimuli.

The linear increase in flicker fusion frequency with the logarithm of light intensity, according to the Ferry-Porter law, is evident from Fig. 5. Whereas DODT and WADENSTEN found a maximal flicker fusion frequency at 75/sec., a value of 90—95/sec. was found in the present investigation. This difference can presumably be explained by the fact that a matt contact glass was used in this work so that uniform illumination of the whole of the retina, or at least of the greater part of it was achieved. As can be inferred from Fig. 5, the flicker fusion frequency diminishes at very high light intensities (above 2,500 lux). In subjective examinations, the same decrease in flicker fusion frequency is obtained in extremely strong light, the subject then stating that he becomes dazzled, as described by HECHT and VERRIJP (1933).

On stimulation with very strong light, the individual flicker potentials exhibited a markedly distinct *a*-wave, but no other noteworthy features.

Discussion.

If the human flicker ERG is compared with that of the pigeon, as recorded by DODT and WIRTH, the striking resemblance is apparent, despite the difference between the proportion of rods and cones in the pigeon and in man. In both cases there is one *a*-wave in two steps (Fig. 2), a three-peaked *b*-wave and a double, positive off-effect. Since the pigeon's retina is known to contain mainly cones (SCHULTZE 1866), this lends support to the assumption that the human flicker ERG on stimulation with strong light is a cone response. The basis of the different ERG phases presumably consists of definite excitatory and inhibitory courses, as indicated to some extent by the experience of other workers.

According to GRANIT (review, 1947), the *a-wave* is an expression of a *preexcitatory inhibition*, which inhibits both the spontaneous activity of the optic nerve and the preceding off-effect. The *a*-wave increases in size with light intensity, and this is the probable cause of the decrease in flicker fusion frequency to lights above 2,500 lux. The *a*-wave also increases with increasing dark adaptation and, under these conditions as well, there is diminishing flicker fusion frequency (DODT 1956).

Since the *b-wave* can arise through *excitation* of both cones and rods, a double *b*-wave is obtained in a mixed retina under certain conditions (GRANIT and MUNSTERHJELM 1937, ADRIAN 1945, MOTOKAWA and MITA 1943, SCHUBERT and BORNSCHEIN 1952, ARMINGTON and BIERSDORF 1956). This duplicity does not, however, explain the splitting of the *b*-wave observed in the present investigation. For example, the peak time for the scotopic *b*-wave given in the literature is considerably longer than the 40 msec. exhibited maximally by the 3rd positive potential in my records. Consequently, it seems probable that, under these conditions, it is a question of a purely photopic *b*-wave, *i. e.*, a cone response. The different peaks in the positive wave can possibly be explained by the difference in latency of various colour components, as in the case of the off-effect (DONNER 1950, MOTOKAWA 1950, GOTO and TOIDA 1954).

Finally, the *off-effect* is an expression of the processes occurring in the retina when illumination ceases. The double off-effect can presumably be explained in the same way as the splitting of the *b*-wave (see above).

Summary.

An account is given of the human photopic flicker electroretinogram (ERG) with increasing flicker frequency, and its different phases are analyzed. With rising frequency, the amplitude initially diminishes owing to interference between the negative *a*-wave and the 2nd positive off-effect making the ERG practically diphasic. With a further increase in flicker frequency, a considerably greater amplitude of response is recorded, owing to superimposition of the off-effect on the *b*-wave. This is followed by a linear decrease in amplitude until fusion occurs. Slightly before fusion, the flicker ERG consists of an *a*-wave and the 1st off-effect.

The flicker fusion frequency is directly proportional to the logarithm of light intensity (Ferry-Porter law) up to 90—95 flashes/sec. With an increase in light intensity above 2,500 lux, the flicker fusion frequency once more diminishes.

The different phases in the flicker ERG are discussed. It is suggested that the splitting of the *b*-wave and the off-effect can be explained by the presence of colour components with different latency periods.

Acknowledgement.

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Components of the Human Electroretinogram.

An Analysis in Normal Eyes and in Colour Blindness. Preliminary Report.

By

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Received 23 January 1957.

Closer analysis of the electroretinogram (ERG) showed at an early stage that the positive *b*-potential exhibits a "staircase effect", and must therefore contain several components. Research in this field — extensively reviewed by GRANIT in his two monographs (1947, 1955) — showed that this was chiefly due to the difference in the rate of reaction of the cones and rods, and that photopic and scotopic potentials could thus be distinguished in the *b*-wave. The first studies were made in animals, whereas the basic observations in man were published by MOTOKAWA and MITA (1942) and independently of them by ADRIAN (1945). It was found relatively easy in man to record a photopic *b*-potential (the so-called *x*-potential), particularly on stimulation with red light. This potential has since been studied in detail by several workers (SCHUBERT and BORNSCHEIN 1952, ARMINGTON 1952, 1953, 1955, ARMINGTON and THIEDE 1954, AUERBACH and BURIAN 1955, ARMINGTON and BIERSDORF 1956). ARMINGTON (1952), found a photopic spectral sensitivity curve, but with an additional sensitivity maximum at 630 mμ. In these studies, it was not possible to demonstrate any additional photopic, positive *b*-potentials with another spectral sensitivity.

The potential observed was best apparent on stimulation with red light, and the aforementioned investigations have therefore been complemented by ERG recordings in colour-blind persons. MOTOKAWA and MITA (1942) obtained an *x*-potential even in protanopes on stimulation with red light, and the same result

was recorded by RENDAHL (in press). Other workers were, however, unable to record any photopic *b*-potential in protanopes under these conditions (SCHUBERT and BORNSCHEIN 1952, ARMINGTON 1952, FRANÇOIS, VERRIEST and DE ROUCK 1956). ARMINGTON stated that this might be explained by insufficient intensity of the stimulus light, since protanopes are known to exhibit decreased sensitivity to light in the long-wave region of the spectrum.

An alternative explanation of the discrepant results will be suggested in the following discussion.

In totally colour-blind persons (monochromatic rod retina) the photopic ERG is entirely lacking (VUKOVICH 1952, YONEMURA and ISHIZAKA 1952, DODT and WADENSTEN 1954, WADENSTEN 1954).

The previously mentioned "staircase effect" in the *b*-wave has, however, been observed even in animals with a pure cone retina (GRANIT 1947). This phenomenon has recently been seen in ERG curves of the pigeon, published by DODT and WIRTH (1954).

In records of the ERG in man, it has now also proved possible to obtain an ERG in which the *b*-potential consists of several components (RONCHI and GRAZI 1956, HECK, in press).

No detailed analysis has hitherto been published of these different components of the *b*-potential, but it is easily conceivable that electric responses have arisen in different kinds of colour-sensitive cells in the retina. Further support of such an assumption is given by the investigations of GOTO and TOIDA (1954) on the off-effect in the frog ERG, in which they showed that the *d*-wave was made up of several components, which exhibited a varying sensitivity to colour.

On the basis of this argument, we have studied the ERG of the human eye in the light-adapted state. We have also attempted to analyze the various partial components in normal eyes, as well as in some colour-blind persons, on stimulation with slow flicker and after adaptation to monochromatic light of varying wave-length.

Methods.

The ERG was recorded with a contact-glass electrode according to KARPE (1945). In order to obtain even illumination of as large an area as possible of the retina, a contact glass covered with a thin layer of fabric ("matt glass") was used. The potentials were amplified by a

condenser-coupled amplifier with time constants of 7 and 70 msec. Each curve was recorded on a stationary film with synchronization over a cathode-ray oscillograph of Cossor type. Each recording implies photography of about 10 superimposed ERGs.

A Philips Tungsten Ribbon lamp 6 V/16 amp. with a colour temperature of 2,800° K, was used as stimulus light and for simultaneous light adaptation. An electromagnetically regulated shutter system gave a constant flicker with 1 stimulus per second (same interval between light and dark). The light intensity was 250—300 lux, measured at the site of the eye, directly behind the contact glass.

In some experiments, the experimental subject was first exposed to adaptation to coloured light (220 V/500 W lamp, interference filter) with maximal transmission at 658 and 549 mμ respectively. The energy of these lamps was not, however, the same, the green light being about three times stronger than the red.

Experimental Subjects.

Normal eyes: two men and two women with clinically normal eyes and normal colour sense (tested with pseudo-isochromatic charts and Nagel's anomaloscope).

Protanopia: three men with clinically normal eyes and typical colour sense defect (tested as above).

Deuteranopia: one man with clinically normal eyes and typical colour sense defect.

Deuteranomalopia: one man with clinically normal eyes and typical colour sense defect.

Achromatopsia: two men with the diagnosis made earlier (by another ophthalmologist). In both cases there was rod monochromasia.

Results.

1. Normal Eyes.

A typical ERG obtained with this method of recording is shown in Fig. 1 (10 superimposed curves from the same subject). The same type of curve was obtained consistently in all of the subjects with normal eyes.

A schematic representation of the same curve is given in Fig. 2. After a latency of 10 msec., the first negative potential (a_1) appears and reaches its deepest point after 20 msec. Under certain conditions (*vide infra*), a_2 follows: it reaches its deepest point after 30 msec. The curve obtained thus exhibits four positive deflections, denoted in Fig. 2 by 1, 2, 3 and 4 with a peak latency of about 27, 33, 40 and 47 msec., respectively.

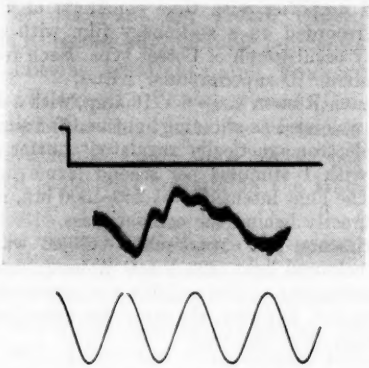

Fig. 1. ERG in man on stimulation with flickering light, intensity 250 lux, frequency 1/sec. (same interval between light and dark). Only the on-effect is seen. The curve shown comprises 10 superimposed records. Uppermost line: light marking. Middle curve: ERG. Bottom curve: time marking 50/sec.

When flicker frequency is altered, this changes the period of the stimulus light, and possibly also the state of adaptation of the eye. At a lower frequency, a_2 appears more distinctly, whereas the 2nd, 3rd and 4th positive deflexions show a tendency to become confluent, owing to the 3rd wave increasing in size and dominating the curve. If, on the other hand, the rate of flicker is increased, the 2nd positive deflexion instead becomes particularly conspicuous.

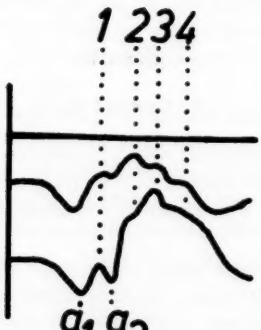

Fig. 2. ERG with different flicker frequencies. Uppermost line: light marking, starting with on. Middle curve: ERG at 250 lux flickered 1/sec. (as in Fig. 1). Bottom curve: as in Fig. 1, but only one light stimulus (duration 0.5 sec.) every 10 seconds.

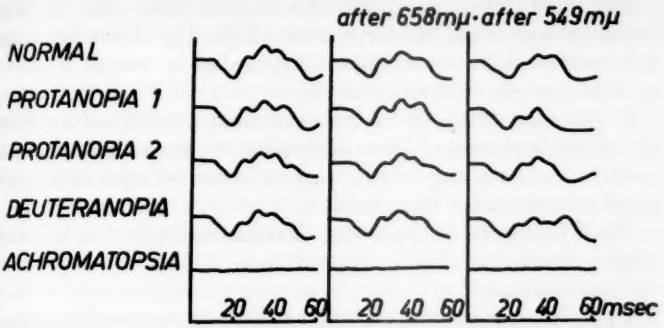

Fig. 3. On-response in flicker ERG, intensity 250 lux, frequency 1/sec. Row 1: normal eyes. Row 2: protanopia, first type. Row 3: protanopia, second type. Row 4: deuteranopia. Row 5: achromatopsia. — Stimulus light white in every case, before and after pre-adaptation to coloured light as indicated.

2. Normal Eyes after Adaptation to Coloured Light.

Pre-adaptation to coloured light was used in an attempt to induce transient suppression of the retinal sensitivity to the adaptation colour used. The subsequent examination, as before, was made with flickering white light (Fig. 3, uppermost row of three curves).

After adaptation to *red light* (658 mμ) the 3rd positive wave was absent for 15—20 sec., whereas the peak time of the 4th wave was slightly shortened. The latency of the 4th wave was afterwards normalized and the 3rd positive wave finally reappeared.

Again, after adaptation to *green light* (549 mμ) the 4th positive wave was absent for the first few minutes. In other respects, the appearance of the curve was normal.

3. Colour Blindness.

a) *Protanopia.*

On examination of three protanopes, two types of change in the ERG were recorded.

The first two subjects exhibited an ERG in which the 3rd positive deflexion was lacking (Fig. 3, second row). This ERG was identical with that of normal eyes adapted to red light. When the eyes of the protanopes were adapted to red light, no change took place in their ERG. On adaptation to green light, the 4th positive deflexion disappeared, as in normal eyes.

The third protanope exhibited a normal ERG with all four positive components. However, after adaptation to red light, the 3rd positive deflexion did not disappear, as in normal eyes.

b) *Deuteranopia, deuteranomalopia.*

In this case, the patient with deuteranopia exhibited an ERG of normal appearance. After adaptation to green light, the 4th positive wave was augmented, whereas in normal eyes under such conditions this wave disappears.

The ERG of the subject with deuteranomalopia was in every respect similar to that of normal eyes.

c) *Achromatopsia.*

Under the stated experimental conditions, two totally colour-blind subjects exhibited no ERG. On dark adaptation, however, a low, normal scotopic (clinical) ERG was obtained. On testing their critical flicker fusion, this was found to lie at 18/sec. (at 30 lux), *i. e.*, a purely scotopic flicker response was elicited.

Discussion.

Thus, on recording the ERG in light-adapted eyes, a photopic *b*-wave was found, made up of several components. Two of them could be studied in greater detail. The earlier of them showed distinct red sensitivity, and seemed to be identical with the potential described by other workers on stimulation with red light. Our investigations are in agreement with previous experience in showing that this potential is lacking in two protanopes. In a comparison with investigations on the off-effect in animals, the red response can also be expected to exhibit the shortest latency.

Our results also explain the additional sensitivity maximum found by ARMINGTON (1952) in the photopic *b*-wave in investigations of the spectral sensitivity. To stimulation with red light, he probably recorded a red component which, owing to its shorter latent period, appeared earlier than the other photopic *b*-potential. With slow flicker frequency, we were also able to observe that, under certain conditions, the red-sensitive component can be the highest point of the photopic *b*-wave.

Since this red component forms only a part of the photopic ERG, it is understandable why certain workers have found a normal photopic *b*-potential (*x*-potential) even in protanopes. Our investigations in addition showed that a protanope (in whom there was no reason to suspect optic nerve damage) exhibited an

ordinary red component, but that this was unaffected by pre-adaptation to red light. This is in contrast to the findings in normal eyes, in which — under such conditions — this positive component of the ERG temporarily disappears. Evidently, at least two types of protanopia exist of which the latter type seems to have normal colour receptors but possibly abnormalities in the optic pathways or centres.

This red-sensitive potential is followed by a last positive wave, but the investigations showed that this is not sensitive to green only. It is known from studies of the off-effect in the frog ERG that a blue component may be concealed in the green response, since the latency period of the two components is highly similar. Further analysis necessitates more detailed investigation with light of shorter wave-length and a spectrum of equal energy.

Records from a deuteranope showed that the "green-sensitive" potential, though seemingly normal, did not, as in normal eyes, disappear on adaptation to green light.

These positive potentials are preceded by two negative deflexions. In the light of earlier analyses (ARMINGTON, JOHNSON and RIGGS 1952, SCHUBERT and BORNSCHEIN 1952, AUERBACH and BURIAN 1955) these should represent photopic and scotopic negativity. Moreover, the latter negativity increases strikingly with decreasing light adaptation. On recording in two totally colour-blind patients, the whole ERG, including both negative components was, however, entirely lacking under our conditions of examination. The clinical (scotopic) ERG nevertheless contained a *b*-potential, which was possibly below the normal range of variation. In a study of achromatopsia VUKOVICH (1952) found that only the first negative wave disappears. It is not at present possible to determine whether our results should be interpreted as an indication that both negative components are of photopic origin, or be assumed to signify that the colour blindness is a link in a more generalized disease of the retina (*cf.* BJÖRK, LINDBLOM and WADENSTEN 1956) with an affection of the retinal rods as well.

Summary.

1. On stimulation with strong flicker of equal light and dark intervals and 1 stimulus/sec., it has been found possible in man to obtain an electroretinogram (ERG) with two negative and four positive waves.

2. In normal eyes, adaptation to red light leads to temporary disappearance of the 3rd positive wave, whereas pre-adaptation to green light enhances the 4th positive wave.

3. The third, red-sensitive component was lacking in two protanopes. This component was present in the third protanope, but was not influenced by adaptation to red light.

4. A deuteranope exhibited the ordinary ERG but, whereas adaptation to red light enhanced the third positive component in the usual way, the ERG was uninfluenced by adaptation to green light. One patient with deuteranomalopia had an ERG similar to that of normal eyes.

5. In two totally colour-blind patients, a photopic ERG was entirely lacking.

6. The red-sensitive component is in agreement with experiences of the photopic ERG published earlier. The 4th positive wave is presumably a photopic function, although a specific green-sensitive component cannot be ruled out. Some properties of the two negative waves are briefly discussed.

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Carbon Monoxide Uptake in Man During Rest and Work.

By

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Knowledge of the uptake of CO in man is of great hygienic importance. Several investigations have been reported of the relationship between the content of CO in inspired air and in blood under various conditions (HENDERSON, HAGGARD, TEAGUE, PRINCE and WUNDERLICH, 1921, SAYERS, MERIWETHER and YANT, 1922, SAYERS, YANT, LEVY and FULTON, 1929, FORBES, DILL, DE SILVA and EDWARDS, 1937, KILLICK, 1936, 1940, FORBES, SARGENT and ROUGHTON, 1945).

The results of these studies, however, were not uniform. For instance, according to a survey by FORBES, SARGENT and ROUGHTON, exposure to 0.02 volumes per cent CO for one hour during light activity entailed COHb values ranging from 6 to 21 per cent. The corresponding COHb ranges after exposure to 0.03 and 0.04 vols. per cent were, respectively, 5.5 to 12.3 and 8.7 to 18.0 per cent.

The importance of the problem and the contradictory results in earlier reports prompted us to study afresh the absorption of inspired CO into the blood. Using a new method, we exposed healthy persons during rest and activity to CO in various concentrations and for various periods. Special efforts were made to

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obtain a reliable method of analysis and constant and accurately controlled conditions in regard to the per cent CO in the inspired air.

General Methods.

The subjects were exposed to CO in streaming air, which was conducted through a plastic hood enclosing the head and neck. The CO content of this air and of the blood, the respiration rate, ventilation, pulse and blood pressure were examined at regular intervals. In the studies during work the O_2 consumption was also determined.

Experimental arrangements: The resting subjects were seated as comfortably as possible in an easychair. In the work studies a bicycle ergometer was used. A known work load was provided by means of an electrically regulated load resistance (HOLMGREN and MATTSSON, 1954). The work performed corresponded to 300, 600 and 900 kgm per minute. To attain satisfactory equilibrium of O_2 consumption, etc., the subject cycled against the desired load for 5 minutes before exposure to CO was begun.

The plastic hood (capacity c. 45 liters) was fitted round the neck with a rubber collar and was suspended from above so as not to weight the shoulders. In all the experiments the rate of flow through the hood was about 50 liters of CO-containing air per minute. The slightly possitive pressure which occurred in the hood prevented suction of fresh air under the rubber collar. For measurement of ventilation a closely-fitting rubber mask was worn over the nose and mouth. This permitted free inhalation of the CO-containing air, while the expired air was led through a wide rubber tube to Douglas bags outside the hood. Each bag could take up large volumes of air with negligible respiratory resistance.

The desired per cent of CO in the inhaled air was achieved as follows. Compressed air and approximately 1 per cent CO were led through separate rotameters (accuracy of readings c. $\pm$ 0.3 and $\pm$ 0.05 liters per minute, respectively) to a glass mixing vessel. The air-CO mixture thereby obtained was conducted through a plastic tube to the plastic hood, whose construction ensured even distribution of the gas. A second plastic tube led from the hood to an evacuation channel.

The CO concentration in the inspired air was checked by systematic analyses, and by observation of the rotameters at intervals of about 3 minutes, with adjustment if necessary.

For the CO analyses we used a meter of the type Stålex SL—2 (see LINDERHOLM and SJÖSTRAND, 1956), to which was connected a writer (Philips). The sensitivity of the apparatus was adjusted so that 0.007 vol. per cent CO corresponded to a reading of 70 to 90 mm, depending on how much the hopcalite filter had been used. This meant that at any rate 0.0001 vol. per cent CO could easily be read. The inhaled air and the calibration gas were examined alternately at intervals of 20 to 30 minutes. Samples of the air were collected between the mixing

vessel and the plastic hood. Before analysis the CO-containing air was diluted with air free from this gas (hopcalite filter) to a calculated CO concentration of about 0.007 vol. per cent, which on the whole corresponded to the concentration of the calibration gas used (see below). In this way analytical errors resulting from possible variation in the sensitivity of the CO meter for different CO concentrations were avoided. The dilution was made by pumping the CO-containing air and fresh air into a rubber balloon through separate, carefully calibrated rotameters (Wobser TG 300). The accuracy of the rotameters was checked[1] with a gasometer constructed for this purpose. Complete conformity within the readability limits of the rotameters was obtained. The CO rotameter could be read with an accuracy of $\pm$ 0.01 liters per minute and the air rotameter with an accuracy of $\pm$ 0.05.

The calibration gas was analyzed[2] for absolute content of CO according to a method described by LINDERHOLM and SJÖSTRAND (1956). Comparison was made with a known quantity of pure CO generated from formic and sulfuric acids as described by HALLBERG (1955). In 25 determinations the calibration gas was found to contain 0.00737 $\pm$ 0.00004 vol. per cent CO. Later in the experiments another calibration gas was used. The CO content was then determined by comparison with the initially used calibration gas; in 11 determinations the mean value was 0.00705 $\pm$ 0.00015 vol. per cent CO.

The CO concentration in the blood was determined with the method of WENNESLAND (1940). Instead of stating the CO in vols. per cent, however, we have recalculated the values to COHb, proceeding from the assumption (see *e. g.* EVANS, 1952) that 1 g of hemoglobin binds 1.34 ml of CO.

Heparinized blood was used. All determinations were made in duplicate or in triplicate. In the double determinations the experimental error was 0.35 per cent COHb and in the triple determinations, 0.29 per cent COHb.

In preliminary studies (BÄCKLUND-LARSSON and FRIBERG, unpublished data) the methods of WENNESLAND (1940) and HORVATH and ROUGHTON (1942) were compared. Fourteen tripled determinations were made with each method within the range 0 to 10 vols. per cent CO. Conformity of results was very good (coefficient of regression 1.009, coefficient of correlation 0.997).

Hemoglobin was measured with a Beckman B apparatus, according to the principle of DRABKIN and AUSTIN (1936). The accuracy of the method was checked by O_2 saturation determinations according to HORVATH and ROUGHTON (1942).

Hemoglobin tests were duplicated. The experimental error was 0.30 g Hb per 100 ml of blood, and was thus 0.21 in the double determinations.

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[1] at the Stockholm Gas Works.

[2] at the Department of Clinical Physiology (Head: Professor T. SJÖSTRAND, M. D.), Karolinska Sjukhuset, Stockholm.

Ventilation was estimated by sucking the collected expired air from a Douglas bag through a flow meter at intervals of 10 to 30 minutes.

The expired O_2 were determined with the method of ENGHOFF (1946).

Experiments at Rest.

The subjects were 10 healthy medical students or laboratory assistants, 3 of whom were women. Their age range was 20 to 28 years, weight 58 to 76 kg and height 169 to 186 cm. Five were smokers.

Exposure was made to 4 concentrations of CO, viz. 0.010, 0.023, 0.040 and 0.080 vol. per cent (table 1, figures 1 to 4). The duration of exposure ranged from 6½ hours at the lowest concentration to 50 minutes at the highest. We aimed to reach COHb readings not exceeding 15 to 20 per cent, so as to exclude all possibility of CO intoxication.

As a rule, a few days at least elapsed before exposure to CO was repeated, but occasionally exposure was made on 2 successive days. After CO exposure, the subject breathed pure oxygen for 15 to 30 minutes.

Variations in the per cent CO in the inspired air were extremely small, both during single experiments and between different experiments of the respective series. Statistical analysis showed that the mean coefficient of variation in single experiments — calculated only for 0.01 and 0.02 vol. per cent CO — was 1.7 per cent for both concentrations. The mean of the individual experiments in each series gave variation coefficients of 1.9, 1.9, 0.8 and 0.7 per cent, respectively, for exposure to 0.01, 0.02, 0.04 and 0.08 vol. per cent CO.

Observations. The CO concentration in the blood was determined before exposure and then at intervals of 10 to 30 minutes, depending on the duration of exposure. The pulse, blood pressure, respiration rate and ventilation were estimated at roughly the same intervals. The hemoglobin was determined before each experiment.

Results.

The results of the experiments at rest are presented in table 1 and figures 1 to 5. The pulse and blood pressure were normal throughout and therefore are not stated.

The CO concentration in the blood showed an almost linear increase with the duration of exposure and a proportionate increase of the per cent CO in inspired air and the increase per unit of time of COHb. For instance, for nonsmokers to reach about 13 per cent COHb when exposed to 0.02, 0.04 or 0.08 vol. per cent CO, a mean period of 200, 100 or 50 minutes was required. After exposure to 0.01 vol. per cent CO for 390 minutes, the COHb

Table 1.

Data (mean values) from experiments at rest.

	Number of persons	CO in inspired air (vol. per cent)	COHb bef. exp. to CO	Respirations per min.	Ventilation (l. per min.)
Nonsmokers	5	0.0100 ± 0.0001	0.5 ± 0.3	17 ± 1	6.8 ± 0.3
	5	0.0230 ± 0.0002	0.6 ± 0.3	15 ± 1	7.0 ± 0.6
	5	0.0403 ± 0.0001	0.9 ± 0.2	16 ± 1	7.0 ± 0.4
	5	0.0798 ± 0.0003	0.4 ± 0.2	16 ± 1	8.0 ± 0.6
Smokers	4	0.0100 ± 0.0001	1.5 ± 0.3	16 ± 1	5.6 ± 0.3
	5	0.0226 ± 0.0002	2.4 ± 0.3	17 ± 2	6.0 ± 0.4
	5	0.0401 ± 0.0001	2.0 ± 0.6	16 ± 1	6.2 ± 0.3
	5	0.0795 ± 0.0002	1.6 ± 0.5	17 ± 2	6.1 ± 0.4

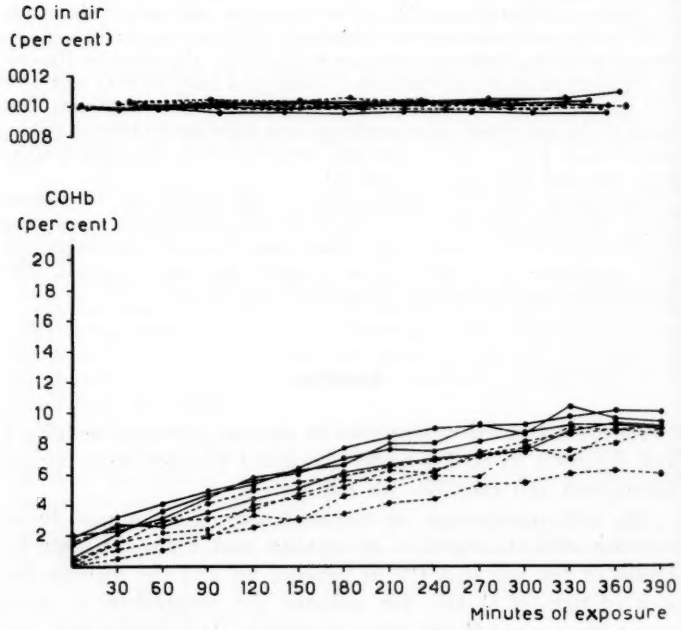

Fig. 1. Uptake of CO at rest (ventilation c. 7 liters per minute) after exposure to 0.01 vol. per cent CO. Smokers (———) and nonsmokers (– – – –).

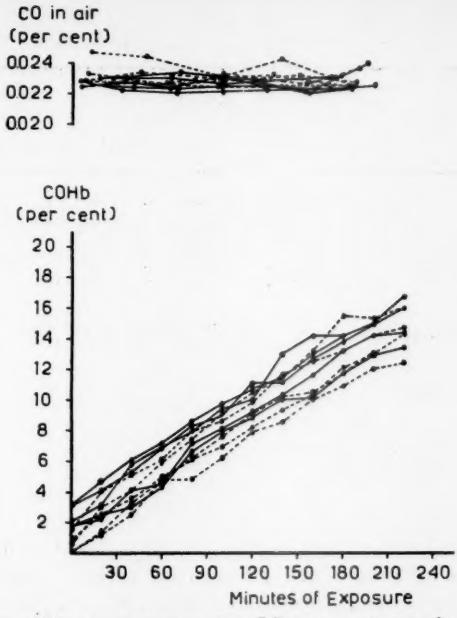

Fig. 2. Uptake of CO at rest (ventilation c. 7 liters per minute) after exposure to 0.02 vol. per cent CO. Smokers (———) and nonsmokers (– – – –).

was approximately 8 per cent. The mean COHb in the smokers was about 1 to 2 per cent higher than in the nonsmokers.

The individual differences are shown in figures 1 to 4. At the end of exposure to CO, the difference between the highest and the lowest COHb was c. 5 per cent, or greater still in the experiments with 0.08 vol. per cent CO.

The spread was to some extent dependent on variations in COHb before exposure (fig. 1—4). As a rule, the higher the initial value the higher the final COHb. This is also apparent in figure 5, in which nonsmokers (initial COHb. c. 0.6 per cent) are compared with smokers (initial COHb c. 1.9 per cent). Even when the initial values were deducted, however, a not insignificant spread remained (c. 2—3 per cent COHb). Random or systematic factors could have been responsible. In the latter case there should be a better correspondence between the final COHb of the same subjects in the 4 exposure series than between all the final readings.

Table 2. *Data (mean values) from experiments at work.*

Work load	Numbers of persons	CO in inspired air (vol. per cent)	COHb bef. exp. to CO	Pulse rate (beats per min.)	
				B[1]	E[2]
Rest	5	0.0230 ± 0.0001	0.5 ± 0.2	74 ± 5	72 ± 7
	5	0.0403 ± 0.0001	0.8 ± 0.2	74 ± 6	66 ± 4
300 kgm per min. .	5	0.0229 ± 0.0002	0.6 ± 0.2	101 ± 4	110 ± 3
	5	0.0400 ± 0.0003	0.6 ± 0.2	106 ± 3	112 ± 4
600 kgm per min. .	5	0.0232 ± 0.0001	0.6 ± 0.2	125 ± 4	135 ± 7
	5	0.0399 ± 0.0002	0.4 ± 0.2	116 ± 4	136 ± 12
900 kgm per min. .	5	0.0232 ± 0.0001	0.6 ± 0.3	150 ± 4	164 ± 8
	5	0.0397 ± 0.0001	0.7 ± 0.1	143 ± 10	153 ± 7

[1] B = at beginning of exposure.
[2] E = at end of exposure.

This would be manifest as less variation "within individuals" from series to series than "between individuals", and would be demonstrable with analysis of variance. An analysis showed, in fact, that factors within the individual subjects produced about half of the total dispersion, and that this circumstance was statistically significant ($P < 0.001$). The spread could not be statistically correlated to variations in exposure or ventilation.

In summary, it can be stated that the individual differences (figures 1 to 4) were attributable to the initial COHb values, systematic factors bound to the individual subjects, and random factors.

Experiments at Work.

The subjects were 5 male students, all nonsmokers. Their age was 19 to 26 years, weight 57 to 70 kg and height 167 to 186 cm.

Exposure was made to 0.023 and 0.040 vol. per cent CO (table 2) for 15 to 70 minutes. The CO in the inspired air was measured immediately before and once during the exposure.

Observations. The CO concentration in the blood was determined before exposure and then at intervals of 5 to 20 minutes. The hemoglobin was estimated before each experiment. The pulse, respiration and ventilation

2.

experiments at work.

Respirations per min.		Ventilation (l per min.)		O_2 consumption (l per min.)	
B	E	B	E	B	E
16 ± 1	15 ± 2	6.9 ± 0.5	7.0 ± 0.4	0.23 ± 0.05	0.23 ± 0.05
16 ± 1	14 ± 1	7.7 ± 0.1	7.2 ± 0.3	0.25 ± 0.01	0.25 ± 0.09
16 ± 1	17 ± 2	20.2 ± 0.6	21.4 ± 0.6	0.85 ± 0.03	0.88 ± 0.02
16 ± 1	16 ± 0	19.6 ± 0.9	19.6 ± 1.1	0.81 ± 0.04	0.85 ± 0.04
18 ± 1	18 ± 1	28.8 ± 0.9	30.3 ± 0.9	1.20 ± 0.02	1.40 ± 0.05
18 ± 2	19 ± 1	26.8 ± 2.2	30.3 ± 2.2	1.20 ± 0.07	1.34 ± 0.08
21 ± 1	26 ± 4	38.7 ± 1.0	42.4 ± 1.7	1.79 ± 0.05	2.00 ± 0.03
23 ± 1	26 ± 3	37.4 ± 2.5	42.7 ± 3.6	1.81 ± 0.06	1.82 ± 0.15

were studied at approximately similar intervals. The O_2 content of the collected expired air and of the inspired air was determined. From the findings and the volume of expired air the O_2 consumption was estimated, taking into account the prevailing temperature and pressure.

Results.

The results of the experiments during activity are presented in table 2 and in figure 6.

The CO concentration in the blood increased with increasing work load (figure 6). In this respect there was no essential difference between exposure to 0.02 and 0.04 vol. per cent CO. The COHb values at rest and with work loads of 300, 600 and 900 kgm per minute showed the approximate relationship 1 : 2 : 2.5 : 3.

The differences between the subjects were of about the same magnitude as in the experiments at rest. The spread was to a great extent caused by individual factors, in that some of the subjects throughout absorbed more or less CO than others. The individual spread did not show significant correlation to variations in exposure, ventilation or O_2 consumption. One of the subjects, however, had exceptionally good ventilation and also showed the greatest absorption of CO.

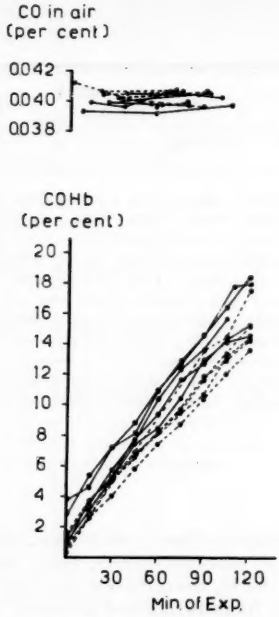

Fig. 3. Uptake of CO at rest (ventilation c. 7 liters per minute) after exposure to 0.04 vol. per cent CO. Smokers (———) and non-smokers (– – – –).

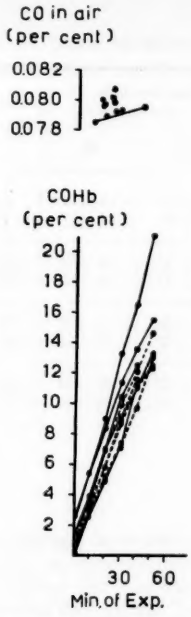

Fig. 4. Uptake of CO at rest (ventilation c. 8 liters per minute) after exposure to 0.08 vol. per cent CO. Smokers (———) and non-smokers (– – – –).

Discussion.

As pointed out in the introduction to this paper, the results of earlier investigations are not uniform. They frequently differ by more than 10 per cent COHb even at the low CO concentrations (< c. 20 per cent COHb) which are most commonly encountered as hygienic problems.

Our average readings in nonsmokers confirm the latest of the previous studies, that of FORBES, SARGENT and ROUGHTON (1945). Because, *inter alia*, of the gradual improvement in the technique of measuring CO in air and in blood, there was *a priori* reason to assume that the report of FORBES, SARGENT and ROUGHTON was the most reliable hitherto. Although our readings generally are very slightly higher than theirs, the difference may undoubtedly be attributed to the fact that, unlike these workers, we did not

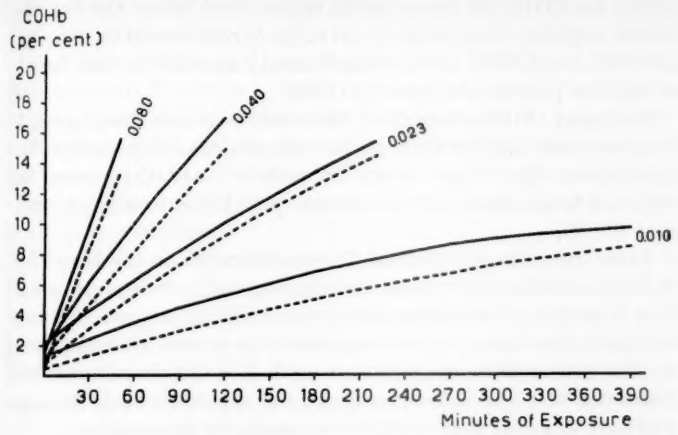

Fig. 5. Uptake of CO (mean readings) at rest after exposure to 0.010, 0.023, 0.040 and 0.080 vol. per cent CO. Smokers (———) and nonsmokers (- - - -).

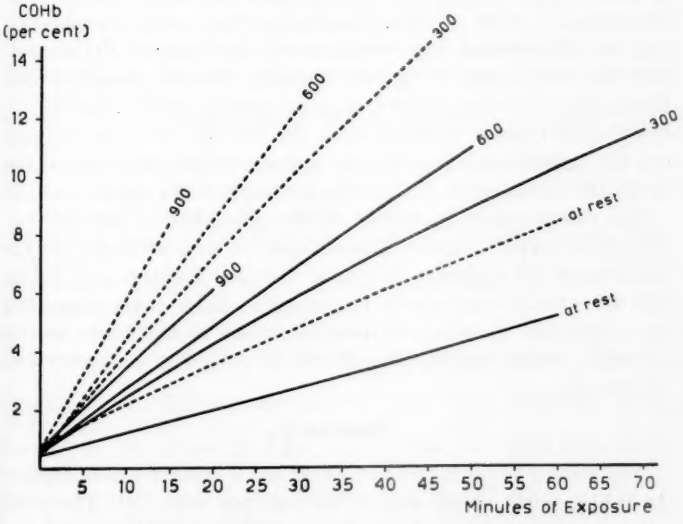

Fig. 6. Uptake of CO (mean readings) in nonsmokers at rest, light (300 kgm per minute), hard (600 kgm per minute) and very hard (900 kgm per minute) work after exposure to 0.023 (———) and 0.040 (- - - -) vol. per cent CO.

correct the COHb for traces of CO in the blood before the experimental exposure. Such traces in our subjects represented on average c. 0.6 per cent COHb, a value which largely agrees with that found in healthy persons (SJÖSTRAND, 1949).

The mean COHb readings of the smokers in our study were 1 to 2 per cent higher than in the nonsmokers. Our curves for nonsmokers thus show values somewhat lower than may be expected from exposure of an average population to a known per cent of CO.

Even after correction of the CO concentration in the blood for the initial value, the spread between our subjects was so great that it could not be disregarded from a practical aspect. FORBES, SARGENT and ROUGHTON did not state the spread after exposure to the various CO concentrations used. Nor did they report the frequency of blood and air analysis. Consequently, their findings and ours unfortunately are not comparable in this respect.

The constancy of the experimental conditions in our study precluded varying exposure to CO as a cause of the individual spread. On the other hand, analysis showed that the spread was to some extent attributable to individual systematic factors. The fundamental cause of this association was not demonstrated; thus in only one case was there a connection between CO absorption and ventilation or O_2 consumption. FORBES, SARGENT and ROUGHTON, who also report that some persons rather consistently absorbed CO faster or slower than the average, however, pointed out the importance of variations in the diffusion constant of the lungs and in the ratio of the tidal air to the dead space.

The discrepancies in earlier reports have led to lack of confidence in many hygienic assessments of and attitudes to the problem of CO exposure. We hope that our findings will reduce this uncertainty and enable hygienists to judge more accurately the important question of the CO uptake in the blood during exposure under varying conditions to different concentrations of the gas.

Summary.

Ten resting subjects (5 nonsmokers and 5 smokers) were exposed to 0.010, 0.023, 0.040 and 0.080 vol. per cent CO. The work studies were made on 5 nonsmokers. They breathed 0.023 and 0.040 vol. per cent CO at rest and during work (bicycle ergometer) corresponding to 300, 600 and 900 kgm per minute.

Results.

The mean COHb readings confirmed the findings of FORBES, SARGENT and ROUGHTON (1945) in resting and in active subjects (figures 5 and 6). Our values were slightly higher (c. 0.5 per cent COHb) but we did not, like these writers, correct our initial readings for traces of CO in the blood before the experimental exposure.

Smokers showed COHb on average about 1 to 2 per cent higher than in nonsmokers.

The maximum individual spread between the subjects was as a rule c. 5 per cent COHb (figures 1 to 4). It was partly attributable to variations in COHb before the experimental exposure and partly to random factors and individual systematic factors. Particularly in the work experiments it was shown that certain subjects throughout absorbed more or less CO than others. The individual spread could not be statistically correlated to differences in exposure, ventilation or O_2 consumption.

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Gastric Motor Responses to Adrenaline and Noradrenaline after Treatment with a Parasympathicolytic Agent.

By

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Received 26 January 1957.

Experiments on salivary glands of the cat have shown that treatment with atropine gives rise to increased secretory responses to adrenaline (EMMELIN and MUREN 1950, 1951 and 1952, EMMELIN, JACOBSOHN and MUREN 1951, EMMELIN 1952). This increase in responsiveness is in most respects similar to that obtained after section of the parasympathetic nerves to the glands. Since tolerance to atropine develops during prolonged treatment, another parasympathicolytic agent, piperidino-aethyl-diphenyl-azetamid (Hoechst) has been used in later studies on supersensitivity of the salivary glands (EMMELIN and HENRIKSSON 1953, STRÖMBLAD 1956 a and b). This substance, for brevity called Hö 9980, has been shown to have an effect similar to atropine on different preparations including isolated guinea pig's intestine (SCHAUMANN and LINDNER 1951).

Other structures, sensitized to adrenaline after interruption of the parasympathetic nervous supply, might be expected to develop a similar supersensitivity after treatment with a parasympathicolytic agent. Since vagotomy in dogs has been shown to increase gastric motor responses to adrenaline (MUREN 1957 a), it was decided to see whether a similar increase in responsiveness could be obtained by treatment with Hö 9980.

Methods.

A total of 105 experiments were made on 8 dogs, weighing 7—28 kg. Gastric motility and motor responses to drugs were studied at repeated experiments on the same animal. The records were obtained by way of an intragastric latex rubber balloon connected to a bromoform manometer. In 6 of the cases a gastric cannula had previously been installed into the lower portion of the gastric corpus, and the balloon was introduced through the cannula and inflated with 80 ml of air. In the 2 remaining cases the balloon was introduced via a permanent oesophageal fistula (MUREN 1957 c) into the stomach and after inflation the balloon was withdrawn so that a position close to the cardia was secured.

Observations were made before and after induction of anaesthesia (nembutal 30 mg/kg intravenously). During the whole experiment the dog was maintained in a standing or hanging position in a Pavlov stand. Gastric motor responses to intravenous injections of adrenaline and l-noradrenaline were studied. The interval between separate experiments on the same dog varied between 3 and 6 days.

Treatment with Hö 9980[1] was given during six successive days with subcutaneous injection of 1 mg/kg body weight. In most cases this dose was given twice daily (in the morning and in the afternoon).

Vagotomy, made on 5 of the dogs, was performed under nembutal anaesthesia with a transthoracic approach. The vagus nerve trunks were cut between ligatures at the lower portion of the oesophagus; and in addition, the superficial layer of the oesophageal wall was stripped off around the circumference in order to insure interruption of minute vagal fibres.

Results.

Control experiments: Two or more successive experiments were made on each animal before any treatment was given. When the balloon was introduced into the stomach of a conscious dog and inflated the manometer pressure increased to a level varying between 20 and 60 mm of bromoform. More or less regular fluctuations of moderate size (5—30 mm of bromoform) were observed. Adrenaline and noradrenaline almost regularly caused a transient fall in pressure and an inhibition of fluctuations when given intravenously to non anaesthetized dogs.

When anaesthesia was given, the pressure fell promptly and

[1] The drug was kindly supplied by A-B. Webass, Gothenburg.

remained at a constant level with no fluctuations (apart from those caused by respiratory movements). During the following period the responses to adrenaline and noradrenaline were always augmentory, *i. e.* a transient increase in pressure was observed. Increasing doses from threshold up to 5 μg/kg gave well graded responses.

The following description and discussion of results refer to responses obtained during anaesthesia. The observations made on conscious dogs will be subject to special considerations in another paper (MUREN 1957 b).

Treatment with Hö 9980: Preliminar experiments with subcutaneous injection of 1 mg/kg of Hö 9980 showed that this dose abolished the gastric responses otherwise obtained by intravenous injection of mecholyl (metacholine chloride). This effect lasted for at least 5 hours, but the responses to mecholyl gradually reappeared during the following 15—20 hours after injection of Hö 9980. No appreciable effect on the responses to adrenaline and noradrenaline could be observed during the first 24 hours after one single injection of Hö 9980 (1 mg/kg). When, however, this dose of Hö 9980 had been given during 3 successive days, the responses to adrenaline and noradrenaline were considerably larger than those obtained in the control experiments. Since a subsequent period of treatment with two daily doses of 1 mg/kg Hö 9980 was found to increase the responsiveness still further, this dosage was used in most cases. Experiments with estimation of the responses to adrenaline and noradrenaline, were made after 3 and 6 days of treatment. The time interval between the experiment and the last injection of Hö 9980 was in most cases 6—8 hours. In all eight dogs the responses to adrenaline and noradrenaline were considerably larger during treatment with Hö 9980 than before. The increase in responsiveness was evident both as an increase in size of the responses to the various doses, and as a decrease in threshold dose. When the treatment was discontinued, subsequent experiments showed a gradual return in responsiveness, and after approximately two weeks the findings were similar to those obtained in the control experiments.

Vagotomy: Five dogs were subjected to vagotomy 2—4 weeks after treatment with Hö 9980. Experiments made 3 and 6 days after operation showed that an increase of the responses to adrenaline and noradrenaline had developed, thus confirming previous observations on the effect of vagotomy (MUREN 1957 a).

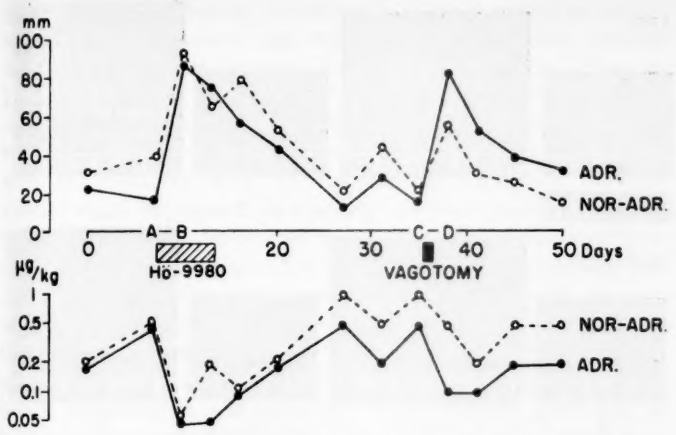

Fig. 1. Repeated experiments on one dog under nembutal anaesthesia. Effect of treatment with Hö 9980 and of vagotomy. Upper curves: Gastric pressure responses (in mm of bromoform) to 2 μg/kg adrenaline and noradrenaline. Lower curves: Motor threshold doses. The letters A—D refer to the experiments shown in Fig. 2.

Figures 1 and 2 show the results obtained from repeated experiments on one and the same dog. The rapid increase in responsiveness and the gradual return after treatment with Hö 9980 is clearly shown for adrenaline as well as for noradrenaline. Similar changes are seen after vagotomy, but these are less pronounced, especially for noradrenaline. Principally the same picture was obtained from the four other dogs subjected to both procedures. In all cases the increase in responsiveness was somewhat less after vagotomy than after treatment with Hö 9980.

In two cases an additional period of treatment with Hö 9980 was instituted 3—5 weeks after vagotomy, at a time when the responsiveness had reached a steady level slightly above the control level. The experiments during this treatment, performed in exactly the same way as during the first period of treatment, also revealed increased responses to adrenaline and noradrenaline. Judging from these two dogs, the level of responsiveness obtained with Hö 9980 was approximately the same after vagotomy as before.

On the average, the size of the responses to adrenaline and noradrenaline was the same on the third and on the sixth day of

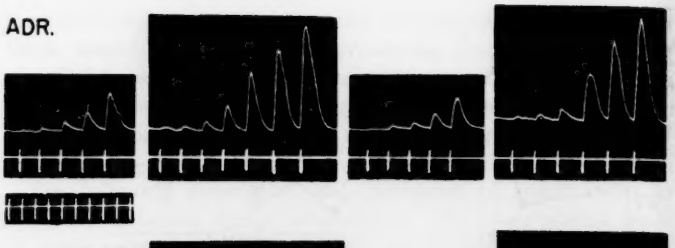

Fig. 2. Records from four different experiments on the same dog as the one presented in Fig. 1. Responses to increasing doses of adrenaline and noradrenaline. A: control; B: during treatment with Hö 9980; C: before vagotomy; D: three days after vagotomy. The injections, indicated by the signals, were given in a standard succession (0.05, 0.1, 0.2, 0.5, 1, 2 and 5 μg/kg), the last signal in each section indicates the injection of 5 μg/kg. The small, separate section on the left gives the time in minutes.

treatment with Hö 9980, *i. e.* increased duration of treatment did not increase the sensitivity further. Treatment extending over longer periods than 6 days, or treatment with bigger doses of Hö 9980 were not performed, due to the fact that the general condition of the dogs was usually influenced by the doses given. One dog died at the end of the period of treatment.

Discussion.

The observation that treatment with a parasympathetically blocking agent gives rise to changes of the gastric motor responses to adrenaline and noradrenaline similar to those observed after vagotomy, indicates that the main cause of these changes is the exclusion of the vagal nervous influence on the gastric muscle cell.

The increase in responsiveness obtained with Hö 9980 was somewhat more pronounced than that observed after vagotomy. Since the experiments with mecholyl indicate that the parasympathetically blocking effect of Hö 9980 was not complete

during the whole period of treatment, there is reason to believe that a further increase of the responses could have been obtained with bigger or more frequent doses of Hö 9980. The difference between treatment with Hö 9980 and vagotomy as to the degree of sensitization obtained, may be due to the difference in peripheral influence exerted by the two procedures. Vagotomy, as well as treatment with an atropine-like substance, prevents central vagal impulses from reaching the plain muscle cell. After vagotomy, which constitutes a preganglionic parasympathetic denervation, the intrinsic nervous system is functionally still intact and local reflexes may reach the plain muscle cells, whereas after "atropinization" the effector cell is deprived of all cholinergic influence. Treatment with Hö 9980 would thus be functionally comparable with a postganglionic parasympathetic denervation which according to Cannon's law of denervation gives rise to a more pronounced sensitization than a preganglionic denervation. Thus, the present experiments support the conclusions arrived at by EMMELIN and STRÖMBLAD (1957) who, in salivary glands of the cat, obtained a higher level of supersensitivity after treatment with Hö 9980 than after section of the chorda tympani.

The decline in supersensitivity observed shortly after vagotomy has previously been attributed to a local nervous activity (MUREN 1957 a). If this conception is correct, a similar return in sensitivity should not be expected to occur during "atropinization". Whether this is actually the case can not be decided on the basis of the present experiments, since treatment with Hö 9980 was not given for longer periods than 6 days.

Summary.

Gastric motor responses to adrenaline and noradrenaline have been recorded manometrically in repeated experiments on dogs. During anaesthesia augmentory responses to these substances were regularly observed.

During treatment with a parasympathicolytic substance (Hö 9980) the responses to adrenaline and noradrenaline were considerably bigger than during the control period.

A similar increase in responsiveness was observed after vagotomy; but the increase was less pronounced after this operation than during treatment with Hö 9980.

The investigation was supported by a grant from the Medical Faculty in Lund.

Technical assistance was given by Miss ASTA HECTOR.

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Influence of the Vagal Innervation on Gastric Motor Responses to Adrenaline and Noradrenaline.

By

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Received 26 January 1957.

According to most reports adrenaline inhibits gastric motility. This effect has been observed mainly in experiments on the intact stomach or on preparations exhibiting peristaltic activity with more or less pronounced muscular tonus. Investigations on isolated preparations from various species has shown that adrenaline may increase or decrease muscular tonus, depending on from which part of the stomach the preparation is taken (SMITH 1918, KURODA 1924, BROWN and MCSWINEY 1926, DUSSARDIER and NAVARRO 1953, WALDER 1953). The augmentory responses were more frequently elicited in preparations from the fundus and corpus than from the distal parts of the stomach.

The dual effect of adrenaline was subjected to special studies by MCSWINEY and BROWN (1926) working with isolated strips from the gastric fundus of dogs and rabbits. They concluded that the type of response depends on the condition of tonus of the gastric musculature, high tonus predisposing to an excitatory response, low tonus to an inhibitory. Subsequent studies (BROWN and MCSWINEY 1932) on the intact stomach of spinal or decerebrate cats showed that a reversal of the adrenaline effect could be obtained by injection of luminal (phenobarbitone sodium). This was attributed to a peripheral action of luminal.

In previous investigations on the delayed effect of exclusion of the vagal influence on the gastric motor responses to chemical agents (MUREN 1957 a and b) the dual action of adrenaline and noradrenaline was clearly apparent and therefore subjected to special studies.

Methods.

Acute experiments were made on 8 dogs, weighing 7—11 kg. The dogs were deprived of food during 24 hours before the experiment. After intravenous anaesthesia with nembutal (pentobarbitone sodium 30 mg/kg) a latex rubber balloon connected to a bromoform manometer was introduced into the stomach via the oesophagus. After inflation with 100 ml of air, the balloon was withdrawn so that a position close to the cardia was secured. Injections of drugs were given intravenously. The stimulation was made of the distal part of the left vagus, sectioned in the neck. Bipolar silver electrodes were used (frequency 0.2—20 stimuli per second, impulse length 2 milliseconds).

Apart from these experiments, results obtained from serial observations made on dogs described elsewhere (MUREN 1957 a and b) are reported in the present paper. These experiments were performed repeatedly on dogs in both the conscious and the anaesthetized state, the balloon being in most cases introduced into the stomach through a gastric fistula. Experiments of this kind were made after transthoracic vagotomy and during treatment with an atropine-like substance (Hö 9980).

Results.

Observations on the dual action of adrenaline and l-noradrenaline obtained from the serial experiments will be described first. These experiments indicated that deviations in manometer pressure corresponded to varying states of contraction of the corpus and fundus. Accordingly, increase in manometer pressure is spoken of as increase in tonus or as an augmentory response and decrease in pressure as decrease in tonus or as an inhibitory response.

Non anaesthetized dogs exhibited more or less regular fluctuations in control experiments. When adrenaline (2 μg/kg) was given, a transient fall in tonus occurred. This was the case in 33 experiments out of a total of 38 experiments on 14 dogs. In the remaining 5 experiments (on 2 dogs) no response or a small increase in tonus was observed. After vagotomy the conditions appeared reversed. Augmentory responses to adrenaline were

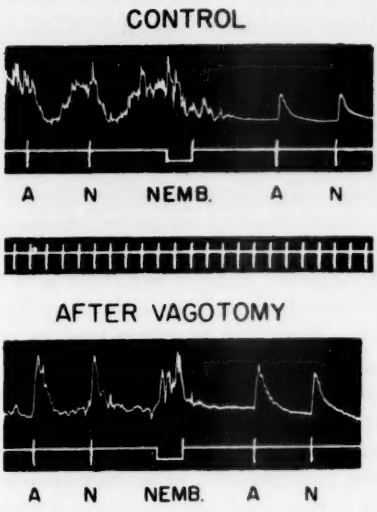

Fig. 1. Gastric motor responses to 2 μg/kg of adrenaline (A) and noradrenaline (N) in the waking and in the anaesthetized state obtained from one and the same dog before and after vagotomy. Time scale in minutes. (The irregular fluctuations during injection of nembutal are due to general agitation).

observed in 27 cases out of a total of 32 experiments on 8 dogs. Similar results were obtained in experiments made during treatment with Hö 9980. Basal gastric activity appeared depressed during treatment with Hö 9980 and during the first few days after vagotomy with no regular fluctuations and a basal tonus somewhat below the control level.

After anaesthesia which induced a fall in manometer pressure and abolished the spontaneous fluctuations (in control experiments), the responses to adrenaline were always augmentory. The size of the responses was increased after vagotomy as well as during treatment with Hö 9980 (Muren 1957 a and b).

With noradrenaline the results were on the whole the same as with adrenaline. With the same doses, the effects were strikingly similar, both regarding size and type of the response.

Fig. 1 gives an example of the typical changes of the record induced by vagotomy. In control experiments the responses are reversed from inhibitory to augmentory ones by the anaesthesia.

After vagotomy (as during treatment with Hö 9980) the record exhibits almost the same picture in the waking as in the anaesthetized state.

These experiments indicate that the augmentory responses to adrenaline and noradrenaline appear when the gastric plain muscle cell is deprived of the normal flow of vagal impulses. Another possible explanation could be that a low tonus is necessary for the augmentory response to appear. The acute experiments described below, may throw some light on these problems.

After inflation of the intragastric balloon in anaesthetized animals the manometer pressure remained at a constant basal level of 10—25 mm of bromoform with no spontaneous fluctuations (apart from those due to respiratory movements). Intravenous injections of adrenaline or noradrenaline gave as usual a transient increase in pressure, the size of which was remarkably constant at separate injections of the same dose in one and the same experiment. When the left cervical vagus was stimulated, the pressure rose and remained at an increased level throughout the stimulation period. This effect usually appeared already at a frequency of 0.2 stimuli/second (one single impulse often gave a distinct response). With increasing frequency up to 1—2 stimuli/second the pressure was gradually increased to a level two or three times the basal value, and regular fluctuations appeared (3—4 fluctuations per second). When adrenaline or noradrenaline was given during this period the response was one of inhibition. During stimulation with low frequencies causing only a slight rise in pressure above the basal level, mixed responses were obtained, with an initial increase followed by inhibition. Stimulation frequencies of 2—20 stimuli/second usually gave irregular responses, showing periods of high as well as low tonus and activity. In order to make sure that the motor responses to vagus stimulation were not caused by the pronounced fall in blood pressure during vagus stimulation in the neck, similar stimulation was made after section of the vagus nerves above the diaphragm. Under these conditions, gastric responses to vagus stimulation were never seen.

Inhibitory responses to adrenaline—noradrenaline during vagus stimulation were regularly observed. When, however, tonus and activity were increased by carbachol (carbaminoylcholine chloride) augmentory responses to adrenaline and noradrenaline were obtained although the pressure had reached a level far

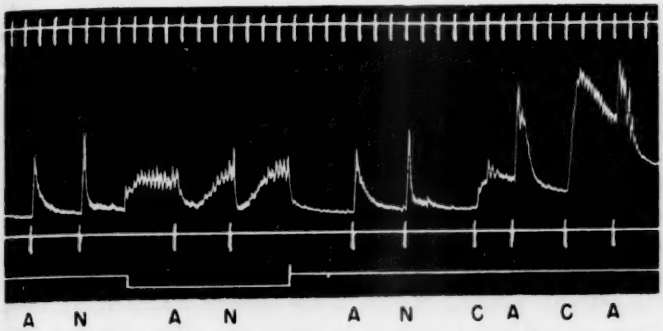

Fig. 2. Gastric motor responses to repeated injections of 2 μg/kg of adrenaline (A) and noradrenaline (N) under nembutal anaesthesia. The period of vagus stimulation is indicated by the lower line. Injections of carbachol (C) 0.5 and 1 μg/kg respectively.

above that obtained by stimulation of the vagus. Fig. 2 gives an example of these phenomena. Augmentory responses were, however, not regularly obtained during stimulation with carbachol. In some cases inhibitory responses to adrenaline—noradrenaline were observed during stimulation with carbachol. These responses were not influenced by intravenous injection of hexamethonium (5 mg/kg).

In some of the experiments, before section of the vagi, the animal was left to regain consciousness to a certain extent. During this period of awakening, the tonus increased and fluctuations appeared until the record showed about the same picture as during stimulation of the vagus. Adrenaline and noradrenaline given during this period elicited inhibitory responses; these were immediately reversed to augmentory ones when nembutal was given. Similar reversal was obtained if atropine (0.5—1 mg/kg intravenously) instead of nembutal was given. Nembutal given after atropine did not change the picture further (apart from abolition of irregularities due to the movements of the awakening dog). When, after section of both vagi, the same degree of consciousness was reached the negative responses were not obtained with adrenaline—noradrenaline. On the other hand, the tonus did not reach as high values as before vagotomy, and no regular fluctuations were seen under these conditions. The appearance of the record was thus similar to that seen after injection of atropine into a conscious dog.

Discussion.

The present investigation indicates that the fundus or the corpus of the stomach is usually relaxed by adrenaline and noradrenaline when the muscle is in a state of high tonus and activity, whereas the responses to the substances are augmentory when the muscle is previously relaxed. These findings are consistent with the statement of BROWN and MCSWINEY (1926) that the reaction depends on the condition of tonus. On the other hand, the actively working muscle is not always inhibited by these drugs, as additional increase in tonus was often obtained with adrenaline—noradrenaline when the stomach was stimulated by previous injection of carbachol. Since inhibitory responses to adrenaline—noradrenaline were observed regularly when the stomach was influenced by vagus impulses, there is reason to believe that these substances interfere in some way with the transmission of impulses from the vagus to the plain muscle cell. When vagal influence on the cell has been interrupted, the responses to adrenaline—noradrenaline are reversed from inhibitory to augmentory ones. Nembutal probably exerts its main action on vagal centres in the brain, as the effect of vagus stimulation was not abolished by nembutal anaesthesia. The existence of a peripheral inhibition exerted by nembutal can, however, not be excluded on the basis of these experiments. The sensitivity of the plain muscle cell to the augmentory effect of adrenaline —noradrenaline is apparently not affected by nembutal since the responses were similar before and after anaesthesia in vagotomized animals (Fig. 1). Atropine, or the related substance Hö 9980, brings about a reversal in conscious animals by blocking the vagus action peripherally. Section of the vagi in the thorax or in the neck has a similar effect. The inhibitory effects of adrenaline and noradrenaline under conditions when the plain muscle cell is stimulated by vagus impulses can possibly be explained as a transient interruption of vagal influence on the cell. The problem is at what point and in what way this interruption takes place.

A centrally depressant effect of adrenaline—noradrenaline could possibly explain the inhibitory gastric responses to these substances obtained on conscious dogs with the vagus nerves intact. Since, however, the present experiments show that the in-

hibitory effect of adrenaline—noradrenaline is readily demonstrable when the sectioned vagus is stimulated artificially, a peripheral blocking action of these substances is undoubtedly involved. Adrenaline and noradrenaline in doses of the same order as those used in present experiments have been shown to inhibit transmission across sympathetic ganglia (MARAZZI 1939, BÜLBRING 1944, LUNDBERG 1952) and a similar action on parasympathetic synapses in the gastric wall may be involved. Apart from a possible effect at this point, however, adrenaline and noradrenaline probably exert an inhibitory influence somewhere between the synaps and the effector cell, since inhibition of the stimulating effect of carbachol was sometimes observed, even after exclusion of the ganglionic effect of this substance with hexamethonium. To what extent vascular effects of adrenaline and noradrenaline are involved in this inhibition can not be ascertained on the basis of this investigation; but the inhibitory effects of adrenaline after pilocarpine and other stimulatory agents observed by BROWN and MCSWINEY (1926) who worked with isolated preparations can hardly be attributed to vascular effects.

Summary.

A large number of serial experiments on dogs have shown that the inhibitory effect of adrenaline and noradrenaline on gastric motility observed in conscious dogs is reversed to an augmentory one by different procedures which interfere with the normal vagal influence such as anaesthesia, vagotomy and treatment with atropine-like substances.

Acute experiments have shown that the augmentory responses obtained during anaesthesia are reversed to inhibitory ones when gastric motor activity is elicited by stimulation of the vagus in the neck. On the other hand, if gastric motor activity is induced by carbachol, the augmentory responses are not regularly reversed.

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The Presence of Acetylcholine in Milk.

By

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Comparatively few studies have been published on the presence of acetylcholine in milk and the classical works from the 1930's do not contain any notes concerning this problem. KEIL and KRITTER (1934) could not find any acetylcholine in cow's milk which had been stored for eight days. Two groups of workers recently announced that the milk from the cow (BEGOVIČ and MAGLAJLIČ 1955 a), the mare (BEGOVIČ and MAGLAJLIČ 1955 b) and woman (HUKOVIČ and HADŽIČ 1955) contain 0.1 to 1 μg of acetylcholine per one ml of milk. In their study biological methods were used to assay the acetylcholine content in fresh or dried whole milk.

The content of choline in human, cow and sheep milk has been investigated more completely, especially by KAHANE and LEVY (1945). The total amount is 160 to 280 mg per litre and only about one tenth of this amount is water soluble. The presence of choline in milk was first announced by MÜLLER (1926).

It is known from the classical work by PLATTNER and HINTNER (1930) that cow's milk has no cholinesterase activity. This has been confirmed with the milk from other animal species including man (AUGUSTINSSON 1948, MCCANCE, HUTCHINSON, DEAN and JONES 1949, MITROVIČ 1955). However, MCCANCE and his group (MCCANCE et al. 1949, HINES and MCCANCE 1953) have made the interesting observation that colostrum from bitches has a high cholinesterase activity. A moderate amount of cholinesterase was also found in sow's colostrum and milk. The physiological

significance of this observation was discussed by McCance and Brown (1953).

In the present study the occurrence of acetylcholine in the milk of cow, woman, and sow was investigated using a technique in which quaternary nitrogen compounds were isolated and then analysed by a paper chromatographic method (Augustinsson and Grahn 1953).

Methods and Material.

Preparation of Extracts.

Cow's milk. Unpasteurized milk and dried milk were used in separate experiments. Ten litres of milk were treated with 1,800 ml of 10 % trichloroacetic acid, kept over night, centrifuged, filtered, and the residue washed twice with 7 % trichloroacetic acid (altogether 4,500 ml). The filtrate was repeatedly extracted with ether, and the volume was reduced to 800 ml by heat (temp. not exceeding 40° C) and in vacuum. During this time the filtrate was shaken with ether twice during the evaporation procedure to remove as much trichloroacetic acid as possible. The final solution (pH 3.5) was filtered and diluted to 1,400 ml.

The spray-dried milk (250 g corresponding to 2.25 litres of whole milk) was shaken three times with ether (to remove the fat), dissolved in 600 ml water, and 340 ml of 10 % trichloroacetic acid were added. The next day the mixture was centrifuged and filtered. After washing the mixture with 7 % trichloroacetic acid the solution was shaken five times with ether (approximately 500 ml each time), evaporated, filtered (to remove sugar) and diluted with the same volume of water. Final pH was 3. One kg of dried milk (corresponding to 8 litres of milk) was treated in a similar way.

Human milk. Two litres of unpasteurized milk were treated with 300 ml of 10 % trichloroacetic acid and then treated in a similar manner as above. The final solution (250 ml) was at a pH of 3.5.

Sow's milk. Altogether 137 ml milk were treated with 22 ml trichloroacetic acid, shaken with ether, evaporated, etc., to give a final extract of 20 ml and pH 3.5.

Isolation of bases as reineckates. The procedure of isolating acetylcholine and other quaternary nitrogen compounds from tissue extracts was described previously (Augustinsson and Grahn 1953, 1954). The water extracts were precipitated with Reinecke salt (2 % aqueous solution), the resulting precipitates were washed three times with ice water, ethanol and ether and then dissolved in aqueous acetone (pH 4). The reineckates of the bases were transformed to the corresponding chlorides by successive treatments with silver sulphate (precipitation of silver reineckate), barium chloride (to remove the excess of SO_4^{2-}). The final solution was reduced to a small volume in vacuum at a

temperature not exceeding 40° C and then tested biologically and chemically, and chromatographed on paper.

Biological assay. The guinea-pig ileum was used as a test object by suspending a 2—3 cm strip in an organ bath (20 ml) of Tyrode solution (according to BENTLEY and SHAW 1952). The bath was oxygenated and maintained at 30° C.

Chemical assay of choline esters was carried out by the hydroxylamine-ferric chloride test (HESTRIN 1949).

Paper chromatography was carried out according to the method recently described (AUGUSTINSSON and GRAHN 1953). The solvent used was a *n*-butanol-ethanol-acetic acid-water mixture (8 : 2 : 1 : 3). Crude protein-free extracts and isolated bases as chlorides were chromatographed, and the chromatograms developed by spraying with dipicrylamine. Appropriate areas (of undeveloped chromatograms), which had been determined by developing a reference chromatogram, were cut out and used directly for bioassay (by putting the strip into the organ bath) or eluted with 96 % ethanol.

Results.

Colorimetric assay of esters. The amounts of esters, expressed in mg of acetylcholine chloride equivalents ("ACh-Cl"), which were present in the solutions of isolated bases are shown in Table 1. These values were obtained with the hydroxylamine-ferric chloride test and showed a content of approximately 1 mg of "ACh-Cl" per litre of milk. This was the result both when whole unpasteurized milk of cow and a spray-dried milk preparation were used as starting materials. In a second experiment carried out with another preparation of dried milk (cow) the content was 0.5 mg per litre of milk. In the case of human milk the total amount of "ACh-Cl" was too low to be estimated with this technique, but acetylcholine was indeed present in the final solution as could be demonstrated with paper chromatography.

Chromatography of bases isolated as chlorides. On the paper chromatograms (Fig. 1) the final solution of isolated bases from cow's milk (whole (B1) and dried (B2)) gave three spots, which were identified as acetylcholine (ACh), choline (Ch) and histamine (Hi). When the acetylcholine area was cut out, extracted and rechromatographed the presence of acetylcholine was confirmed. In addition, there were traces of choline, formed by hydrolysis during the extraction procedure of the acetylcholine area. The chromatographic results obtained with a second dried milk preparation (B3) were similar. When human milk was used as starting

Table 1.

The amount of acetylcholine chloride equivalents ("ACh-Cl") isolated from milk.

Starting material		Final soln. of isolated bases as chlorides ml	Total amount of isolated "ACh-Cl" (chem. assay) mg	mg of "ACh-Cl" per litre of milk (estimated)
Type of milk	Amount (litres)			
Cow's milk, unpasteur. ..	10	10	10	1
Cow's milk, spray-dried ..	2.25 (250 g)	12	2.5	1 (10 μg per g dried milk)
Cow's milk, spray-dried ..	8 (900 g)	11	4	0.5 (4 μg per g dried milk)
Human milk, unpasteur..	2	3.7	biol. assay of chromatograms	

material the presence of acetylcholine in the final solution (B4) could be traced on the paper chromatogram only by bioassay. In this case the two visible spots were identified as choline and histamine.

With sow's milk a detailed analysis could not be performed due to lack of sufficient material. The final solution of isolated base chlorides gave two visible spots on the paper chromatogram and these were identified as choline and histamine. Acetylcholine could be traced with difficulty by the bioassay method.

Bioassay. Acetylcholine was assayed biologically using the isolated guinea-pig ileum as a test object. The solutions of isolated bases were active in all cases, but a quantitative comparison with the data obtained by the chemical assay method was difficult to achieve. For cow's milk and human milk the active isolated principle was destroyed by incubation with purified human plasma cholinesterase and with a sodium hydroxide solution. Similar results were obtained with extracts of the acetylcholine areas of paper chromatograms. The activity of such extracts was completely blocked after the addition of atropine sulphate (0.5 μg) to the organ bath.

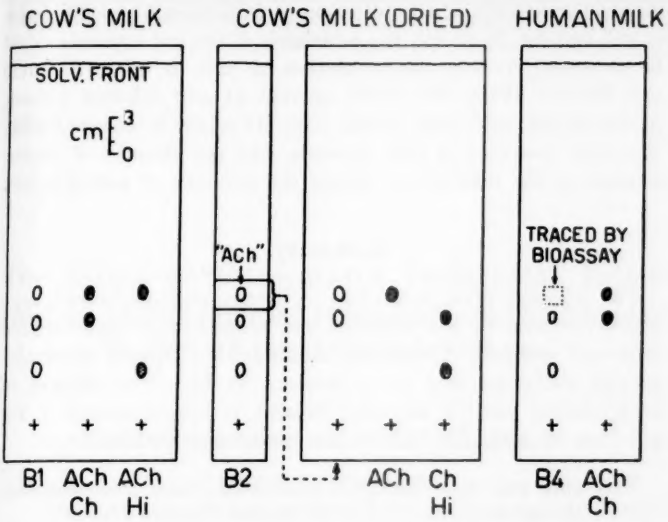

Fig. 1. Ascending paper chromatograms of bases isolated as chlorides from cow's unpasteurized milk (B1), cow's dried milk (B2), and human unpasteurized milk (B4). R_F: acetylcholine chloride (ACh) 0.36; choline chloride (Ch) 0.28; histamine dihydrochloride (Hi) 0.15. Rechromatography of the acetylcholine area ("ACh") of B2 after the elution of the area with ethanol.

The bioassay technique was the only means by which acetylcholine could be determined from the traces on the paper chromatograms of isolated bases from human milk and sow's milk.

Discussion.

The results reported above show conclusively that acetylcholine is present in cow's milk and human milk, and they confirm the recent finding made by the Jugoslavian workers using a less specific technique. The negative results reported previously, amongst others by KEIL and KRITTER (1934), are most probably due to the fact that the preparations used were either stored before tested or the active constituent was destroyed by other means. In all these previous experiments crude preparations have been employed and no isolation procedures have been tried.

The physiological rôle of the acetylcholine present in the milk is unknown. It may have some significance for the normal peri-

staltic movements of the intestine of infants but proof for this is still lacking. However, the peristalsis of the cat intestine could be increased by the administration of milk in situ (Hukovič and Hadžič 1955). The small amount of acetylcholine present in the gastric juice may partly have its origin in ingested milk. The acid reaction of this secretion and the absence of cholinesterase in the milk would favour the stability of acetylcholine.

Summary.

The presence of acetylcholine in human milk and cow's milk was demonstrated. Acetylcholine was isolated from unpasteurized milk and spray-dried milk and identified by a paper chromatographic technique and by a bioassay method. The amount of acetylcholine (as the chloride) present is approximately 1 mg per litre of milk. Sow's milk also contains acetylcholine.

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The Effect of Intracarotid Centripetal Injection of Cholinesterase Inhibitors on the Nuclear Region of the Vestibular Nerve.

By

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In earlier investigations of the central effect of cholinesterase-inhibiting substances, among other aspects, their effect on the central vestibular organ has been studied. It was possible to induce a vestibular syndrome in guinea-pigs by means of intracarotid, centripetal injection of tabun (DIAMANT 1954). In conjunction therewith the cholinesterase activities in the brains of guinea-pigs were investigated. An appreciable decrease in the esterase activity of the pons-medulla region was demonstrated on the side injected. It was indicated that the syndrome probably was induced in the vestibular nuclei in the brain stem. It was impossible, however, to verify statistically a direct relation between the decrease in cholinesterase activity and the pharmacodynamic action.

The following technique was used in these earlier experiments. The brain was divided into four parts; one comprising the pons-medulla region. This part then contained considerably more than the vestibular nuclear region alone. It is conceivable that another kind of division of the brain might better demonstrate the relation between the inhibition due to cholinesterase inhibitors and their effects. It was thus decided to perform a series of experiments founded on a new division technique.

Method.

The investigations were carried out in guinea-pigs as previously described (DIAMANT and TAMMELIN 1953, DIAMANT 1954). Altogether 23 experiments were conducted. A 0.001 per cent solution of tabun was injected in a dose of 0.03 mg/kg. Because of the anatomic conditions in the guinea-pig the injection was always administered on the right side. The animals were killed at various times after the injection, usually immediately, in some experiments however, after $1^1/_2$—2 hours. The brain was removed and divided so that the pons-medulla region, which comprised one part in earlier experiments, was split into three parts. The middle part contained principally the vestibular nuclei as was controlled histologically.

The cholinesterase activity was determined in the cerebellum and pons-medulla region. Only small portions of the different parts of the brain were used. Duplicate determinations were carried out. The activity was determined in corresponding brain parts from uninjected animals too.

In order to facilitate evaluation of the results the animals were classified as follows:

1. Animals in which the injection of tabun induced a vestibular syndrome and which were killed immediately.
2. Animals in which the injection induced a transient vestibular syndrome lasting about an hour. These animals were killed after $1^1/_2$—2 hours.
3. Animals without syndrome after the injection, which were killed immediately.
4. Animals without syndrome after the injection, which were killed after $1^1/_2$—2 hours.

In all these cases the cholinesterase activity was determined within 24 hours.

5. Animals with and without the syndrome after tabun injection. Most of these animals were killed after a period longer than one day. Some animals with symptoms were killed immediately after the injection, but the activity in the parts of the brain was not determined until after at least 48 hours. The preparations were kept in a refrigerator.
6. Untreated animals.

Results.

Group 1, which included six animals, showed a distinct difference in cholinesterase activity between right and left sides (Table 1 A). The activity was considerably lower on the right side, and the difference between the left and right side is statistically significant. The inhibitor affected the entire right side.

Group 2 also presented a definite difference in activity between right and left sides (Table 1 B). It was not so apparent as in Group

Table 1.

Cholinesterase activity in special parts of the brain. A—D after carotid centripetal injection of tabun.

A. The animals got a vestibular syndrome and were immediately killed.
B. The animals got a vestibular syndrome and were killed when this disappeared, *i. e.* after $1^1/_2$ to 2 hours.
C. The animals did not get any vestibular syndrome. They were immediately killed.
D. The animals did not get any vestibular syndrome. They were killed after $1^1/_2$ to 2 hours.
E. Untreated animals. In animal no. 2 the duplicate determinations were carried out with 24 hours interval.

Animal no.		Part of the brain							
		Cerebellum		Medulla oblongata		Vestibular nuclear region		Pons cerebri	
		left	right	left	right	left	right	left	right
A.	1....	0.61	0.14	0.74	0.23	0.75	0.16	—	—
	2....	0.53	0.07	0.34	0.09	0.33	0.08	0.38	0.08
	3....	0.20	0.05	0.25	0.16	0.30	0.10	0.39	0.13
	4....	0.77	0.19	0.56	0.23	0.47	0.20	0.62	—
	5....	—	—	0.59	0.23	0.81	0.26	0.96	0.23
	6....	1.44	0.28	0.88	0.30	0.50	0.22	0.36	0.20
B.	7....	0.67	1.13	0.44	0.31	0.18	0.10	—	—
	8....	0.29	0.23	0.23	0.14	0.38	0.10	0.47	0.10
	9....	0.57	0.55	0.43	0.24	0.36	0.20	0.60	0.38
C.	10....	0.46	0.17	0.29	0.24	0.59	0.19	0.92	0.33
	11....	0.60	0.15	1.01	0.37	1.15	0.27	1.06	0.32
	12....	1.27	1.17	0.63	1.19	0.31	0.55	—	—
	13....	0.20	0.21	0.77	0.18	0.06	0.16	0.16	0.10
D.	14....	0.46	0.22	0.36	0.48	0.65	0.33	0.85	0.49
	15....	0.09	0.11	0.24	0.10	0.20	0.16	0.14	0.07
	16....	1.26	0.80	1.20	0.96	0.74	0.98	0.86	0.70
E.	17....	1.58	1.41	1.78	1.79	1.68	1.30	1.88	1.36
	18....	1.42	0.77	1.00	1.23	1.07	1.09	1.49	1.55
		0.84	0.71	—	—	1.06	1.30	1.37	1.52
	19....	1.89	1.32	1.22	1.42	1.65	1.00	1.30	1.11

The figures mean μ mol HAc/ml · min · g tissue.
In some parts the determinations could not be carried out according to tec hnical or other difficulties. These parts are marked "—" in this table.

1, however, and did not occur in the cerebellum in animals 7 and 9, although the activity in the cerebellum was lower than in the normal animals.

Group 3 showed contradictory results (Table 1 C). In two of the animals the activity was notably lower on the right side. This difference between right and left side did not appear in either of the other two animals. In one of these cases the activity was greatly decreased throughout, with the exception of the left medulla oblongata. In animal 12 the activity was found to be lower in the vestibular region of the left side.

Group 4 comprised three animals (Table 1 D). Animal 14 showed lower activity on the right side although not in the medulla oblongata. In the other two experiments the activity was largely the same in the brain parts from both sides. Animal 15 presented a decreased activity in comparison with normal animals.

It was evident from the experiments in Group 5 (Table 2) that a return to the normal level of the cholinesterase activity can be observed in animals allowed to live longer than two hours after the injection. A delay in the activity determination also resulted in some equalization of the activities of the right and left sides.

The result of determinations of the normal activity showed nearly the same figures as in the paper of DIAMANT and TAMMELIN (1953) (Table 1 E).

Table 2.

Cholinesterase activity in special parts of the brain following intracarotid centripetal injection of tabun. All the animals got a vestibular syndrome.

Animal no.	Killed after injection	Time for determination of activity after death	Part of brain							
			Cerebellum		Medulla oblongata		Vestibular nuclear region		Pons cerebri	
			left	right	left	right	left	right	left	right
1	2 days	1 day	1.45	1.05	1.01	0.47	0.75	0.73	1.37	0.82
2	immediately	4 days	0.89	0.96	0.46	0.30	0.73	0.36	0.68	0.34
3	1 day	4 days	0.85	0.89	1.70	1.07	0.59	0.95	0.39	0.66
4	1½ day	2 days	0.34	0.11	0.31	0.23	0.31	0.16	0.49	0.37

The figures mean μ mol HAc/ml • min • g tissue.

Discussion.

The discussion of the effect of cholinesterase-inhibiting substances has consistently centered about the relation between inhibition and pharmacodynamic effect. Many have considered that the

results of animal experiments indicate a definite connection (FREEDMAN, BALES, WILLIS and HIMWICH 1948, SCHIFF, ESMOND and HIMWICH 1952, KAMIJO and KOELLE, 1952, APRISON, NATHAN and HIMWICH 1954). DIAMANT (1954) has demonstrated a degree of conformity between the decrease in the cholinesterase activity and the severity of the induced vestibular syndrome. The conformity was not entirely conclusive, however, and it was pointed out in the conclusions drawn that the possibility of a direct pharmacodynamic effect could therefore not be excluded.

If the cholinesterase inhibition is the cause of the symptoms, it should be possible to demonstrate a relation between inhibition and effect. Our experimental results do not unequivocally permit this. However, if we analyse the results, we find that the deviations can be explained. In the group of animals in which symptoms are induced directly and followed immediately by death (Group 1) there is always a clear difference in activity between the right and left sides such as was also shown in an earlier investigation (DIAMANT 1954). The depression of the activity is always on the right side. The results are largely the same if the animal is not killed until after the syndrome has subsided (Group 2). A certain equalization of the activities is apparent, however, indicating that a comparatively rapid restitution of the cholinesterase activity takes place in the brain. This is also clearly evident in Table 2, animal 1, where an interval of two days elapsed before the animal was killed. In this case the cholinesterase activity was on the way up towards "normal". A study of the phosphoryl phosphatase content of the different parts of the brain would be interesting in this connection.

Thus, while the results in Groups 1 and 2 may well agree with the theory that the effect of cholinesterase inhibitors is due to enzyme inhibition, it is more difficult to explain the results in Group 3. There two of the four cases showed a definite decrease in the activity on the right side in comparison with the left. By rights, vestibular symptoms should have occurred in these cases. That this did not take place may depend upon insufficient difference in activity between the two sides in these particular animals for the induction of a syndrome. However, there are other factors affecting the induction of the syndrome. A factor like this is for example different individual sensitivity against organo-phosphorous compounds. This permits an explanation of failure of syndrome to occur.

Of the three animals in Group 4 two exhibited similar activity in the different parts of the brain. If this similarity was initially present or resulted from an equalization cannot be established with certainty. In the third animal there was partially lower activity on the right side. In the first two animals the absence of a syndrome may have been due to the lack of any difference in activity between right and left sides. In the third animal perhaps the difference was too small. However, the earlier-mentioned restitution of the cholinesterase activity must be taken into consideration.

Accordingly, the foregoing experiments do not preclude the possibility of a direct pharmacodynamic effect of cholinesterase inhibitors. Perhaps, when satisfactory histochemical methods for quantitative determination of the cholinesterase activity have been developed, it will be possible to determine with greater certainty what occurs within the central vestibular region.

Summary.

Using an earlier-described technique in which a vestibular syndrome is induced by means of intracarotid, centripetal injection of tabun the writers attempt to throw light on the question of the cause of the pharmacodynamic effect of cholinesterase inhibitors in vivo. The results show a definite relation between the induced syndrome and reduced enzyme activity. In some cases where the syndrome does not occur, there is nevertheless a definite unsymmetric decrease of the cholinesterase activity in the brain with lower activity on the injected side. It is therefore impossible to establish definitely that it is the enzyme-inhibiting effect of the cholinesterase inhibitors which causes their pharmacodynamic effect. It may as well be a specific pharmacodynamic action. The experiments further show a rapid restitution of the enzyme activity which also occurs in brain preparations kept more than 24 hours in vitro. Determination of the activity shall accordingly be carried out within this period.

Our sincere thanks are due to Prof. G. LJUNGGREN, head of the Research Institute of National Defence, Dept. 1, for his kind interest in this work.

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The Influence of Oxygen Tension and Some Drugs on Human Placental Vessels.

By

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The human placenta has been a subject for investigations since a very long time. Perfusion experiments have usually been performed with different salt solutions (UEDA 1931, v. EULER 1938, ELIASSON and ÅSTRÖM 1955 inter alia). In a few investigations animals blood was used (RECH 1924, BUDELMANN 1929).

Placental perfusions with human blood were performed in 1929 by KÜSTNER and SIEDENTOPF. No consideration was taken to blood groups. Citrate solution was used to prevent coagulation. The blood was stored for different periods of time and a considerable leakage of potassium from blood cells to plasma may therefore have occurred. Probably partly dependent on different experimental methods the results were not all in agreement.

In 1922 SCHMITT observed constriction of placental vessels occurring with a few minutes interval. This observation was recently confirmed by ELIASSON and ÅSTRÖM (1955).

The oxygen consumption of the placenta was estimated by KÜSTNER and SIEDENTOPF (1929) to 12 ml O_2 per kg and minute. BUDELMANN (1929) using defibrinated dogs blood estimated the placental oxygen consumption to about 2 ml per kg and minute.

The effect of oxygen to the placental vessels was studied by SCHMITT (1922) upon isolated vessels immersed in blood serum and by UEDA (1931) upon the whole placenta perfused with salt solution. Oxygen caused contraction and carbon dioxide caused dilatation of the vessels.

Histologically the wall of the fetal vessels of the placenta has been found by the majority of investigators to have three muscular layers. The circular musculature is on both sides surrounded by a layer of longitudinal muscle fibers. There is no essential difference between artery and vein in this respect but the artery has a relatively thicker circular layer (LOCHMANN 1900).

In spite of many histological investigations one has not yet been successful in finding any nerves in the placenta or in the distal part of the umbilical cord (SCHMITT 1922, BAUR, RUNGE and HARTMANN 1929).

The effects of different drugs have been tested on placental vessels by several investigators.

The effect of adrenaline was studied among others by SCHMITT (1922), KOSAKÁE (1927), KÜSTNER and SIEDENTOPF (1930), UEDA (1931), v. EULER (1938), DAWSON and ROBSON (1940) and DORNHORST and YOUNG (1952). In a recent investigation (ELIASSON and ÅSTRÖM 1955), adrenaline and noradrenaline were found to cause a vasoconstriction which confirmed some of the previous investigations.

Acetylcholine was found to cause vasoconstriction as well as vasodilatation, partly dependent on dosage (UEDA 1931, v. EULER 1938, ELIASSON and ÅSTRÖM 1955).

Papaverine was found to cause vasodilatation (ELIASSON and ÅSTRÖM 1955).

In perfusion experiments on cats DAWSON and ROBSON (1940) studied the effect of posterior pituitary extract on the placental circulation. There occurred a vasoconstriction of long duration.

As perfusion experiments with salt solutions, animals blood or citrate human blood may cause errors, we considered it of some interest to investigate the human placenta under more physiological conditions. We considered it necessary to use heparinized blood in order to avoid hypocalcemia and disturbances in the buffering capacity of the blood. The blood must also be fresh, otherwise hemolysis and leakage of potassium from the blood cells to plasma might cause errors.

Materials and Methods.

The placenta.

The experiments were performed in the delivery department, which minimized the period between delivery and the beginning of perfusion. Placentas from normal deliveries only were used. The mothers had no hypertensive disease or albuminuria. The maternal surfaces of the placentas were intact and without macroscopic changes. The placenta was kept in 37° C Ringer-glucose solution to prevent drying and cooling.

The umbilical cord was cut a few centimeters from the placenta in order to permit the two cannulas, inserted in the umbilical vein and one artery, to enter the placental vessels. The other umbilical artery was ligated. The fetal blood was then washed out by perfusing the placenta with Ringer-glucose solution aerated with 6.5 per cent carbon dioxide and 93.5 per cent oxygen. This solution contained 5 mg heparin per 100 ml. During the washing out of fetal blood a few small clots usually appeared in the outflow. The perfusion with salt solution was stopped when the outflow was clear. Thereafter the perfusion with human blood begun. Thanks to the anastomoses at the umbilical insertion both halves of the placenta were equally perfused.

The blood.

The donor blood was withdrawn from a cubital vein of a male 1—2 hours before the experiment. When two donors were used the blood was mixed and divided into two or three equal portions. Rh-positive 0-blood containing 10 mg heparin per 100 ml was used in every experiment. In order to obtain blood of different oxygen saturation it was aerated with pure oxygen or nitrogen for constant periods of time. Oxygen and carbon dioxide tensions were determined according to the method of Björk and Hilty (1954). Duplicate determinations showed an error of 2 per cent. Mean values for the three types of blood used (oxygenated, venous and hypoxemic) are indicated in table I. During the perfusion experiments the surface of the blood in the infusion bottle was covered with liquid paraffin. Sedimentation of blood cells was prevented by occasional shaking. Hematocrite determinations 2—3 hours after beginning of perfusion showed no perceptible change from the initial value. No macroscopic hemolysis was present. Infused blood was passed through two nylon filters. The blood was never reperfused.

The drugs.

The effect of several drugs was tested in the following doses: adrenaline and noradrenaline (exadrin and norexadrin, Astra) 10—20 μg; acetylcholine (Hoffmann la Roche) 5 μg and 1 mg; papaverine 0.4—1.6 mg; oxytocin (utedrin, Astra) 0.1—0.2 I. U.; oxytocin and vasopressin (hypadrin, Astra) 0.1—0.2 I. U. These doses were each contained in 1 ml of normal saline. The pH at 20° C of the different solutions injected ranged from 4.6 to 6.2. The injections were made through an Olofsson rubber membrane needle, connected to the arterial cannula.

Table I.

Type of blood	p O_2 $M \pm \epsilon_M$	p CO_2 $M \pm \epsilon_M$
Oxygenated (O)....	222 ± 55	31 ± 11
Venous	48 ± 1	52 ± 8
Hypoxemic (N)....	17 ± 4	33 ± 10

Experimental Technic.

I. Perfusion with Constant Pressure.

The perfusion pressure was maintained by means of a constant hydrostatic level of the blood. The pressure had to be varied in the different experiments in order to obtain comparable flow of blood in the different placentas. Pressures employed ranged from 40 to 140 mm Hg. The placenta was enclosed in a 4 liter glass holder filled with aerated Ringer-glucose solution. Volume changes and vascular resistance of the placenta were recorded with two mercury manometers. The temperature within the holder was kept constant at 37° C by means of a thermoregulated waterbath. Blood-flow was recorded by an electronic drip counter. Calibration showed an error of 5—10 per cent in the volume measured. The connecting tubes and the cannulas for perfusion were made of nylon (Fig. 1).

II. Perfusion with Constant Output.

In these experiments a modified Gibbon "roller" pump was used. The blood flow was calibrated to 50 ml per minute. The mean pressure was about 60 mm Hg. The experimental arrangement was the same as described in part 1, but only pressure recording was used. The blood was not reperfused. No macroscopic haemolysis occurred in the pump system.

III. Experiments on Isolated Placental Vessels.

In addition to the tests with perfusion of the whole placenta some experiments were performed on isolated and surviving placental vessels. A vein or an artery was dissected out from the fetal surface of the placenta to a length of about 5 cm. In order to test the function of the circular muscular layer only, the vessel was cut to a spiral which was placed in a vertical plexiglass cylinder and suspended as in an ordinary pharmacological bath. The cylinder was continuously infused at a slow rate with oxygenated or hypoxemic heparinized blood to a constant level in the bath above the specimen. Variations in length of the blood vessel were recorded with a level balanced to a traction of the vessel of 2 g in all experiments.

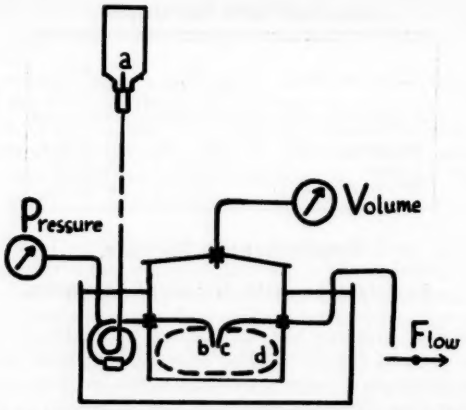

Fig. 1. The apparatus for perfusion of placenta. a = glass jar with blood (or a modified Gibbon pump). b and c = cannulas entering the placenta (d).

Errors.

Slight variations in perfusion pressure during an experiment cause considerable changes in blood flow. Cooling of the placenta or the perfusion solution will often abolish the normal variations in contraction of the placental blood vessels. If the cannulas are inserted into the umbilical vessels significant variations caused by vasoconstriction or kinking can occur. In these studies the cannulas were inserted directly into the placental vessels.

Infused blood must be filtered. In spite of this precaution, pre-existing clots in the placenta may cause total or partial occlusion of the outflow. When plethysmographic recordings are made, leakage from the maternal surface will give false increases in volume. A true increase in volume, due to oedema, occurred when the placenta was perfused with Ringer-glucose solution for some time. When blood was perfused no leakage could be registered and hematocrite determinations of the blood before and after passage of the placenta showed that no leakage of plasma had occurred.

Errors from clots, kinking, leakage and oedema can easily be detected and were mainly observed when volume recording was used (Fig. 2). These illustrated changes in pressure, placental volume and blood flow are always considerable and protracted; they end either with a sudden return to previous conditions or complete occlusion of outflow. The patency of the perfusion system is tested by small injections of saline solution into the arterial cannula. If the result of the injection is a transient increase in pressure, volume and outflow, the system is not occluded.

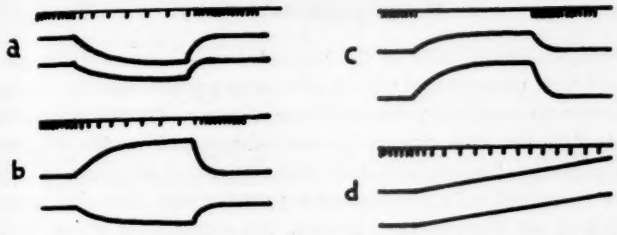

Fig. 2. Examples of errors due to a) obstruction in the afferent tube before the pressure recording device, b) obstruction in the afferent cannula, c) obstruction in the efferent cannula, d) oedema of or leakage from the placenta. The recordings are from above: flow, pressure and volume.

Sedimentation of blood cells may cause some errors by changing the viscosity of the blood, but this error was found to be negligible in the present experiments.

Incompatibility of blood groups must be taken into consideration. The fetal blood lacks agglutinins in its plasma. It would therefore be most adequate to use donor blood of group AB. Large quantities of blood belonging to this group were not available. We therefore chose to use blood of group 0, although the presence of agglutinins in the donor blood might cause agglutination of the fetal blood cells in the placenta. This was prevented by washing out the fetal blood with Ringer-glucose. Microscopical examination of the placental outflow during perfusion experiments indicated that no agglutination occurred.

Finally, leakage of potassium from blood cells must be mentioned, although it was found to be insignificant in these short time perfusion experiments.

From a physiological point of view, placental perfusion experiments are rather unsatisfactory as we cannot introduce a maternal circulation. Conclusions must therefore be drawn with great care.

Results.

I. Citrate Blood.

In 11 experiments with constant output citrate blood was used. The pressure was then mounting continuously until it could no longer be recorded (about 250 mm Hg). It was therefore considered impossible to use that blood in physiological experiments on the placenta. In the experiments recorded below heparinized blood was used.

II. Spontaneous Activity.

Spontaneous activity of the placental vessels causing changes in blood flow, pressure and placental volume was recorded in 9 experiments with constant pressure. These changes occurred at intervals of 1—1½ min. and appeared to be independent of the degree of oxygenation. The main feature was a transient decrease of blood flow combined with an increase in pressure and placental volume followed by the reverse changes. An example is given in fig. 3. This confirms the observations made by SCHMITT 1922 and ELIASSON and ÅSTRÖM 1955.

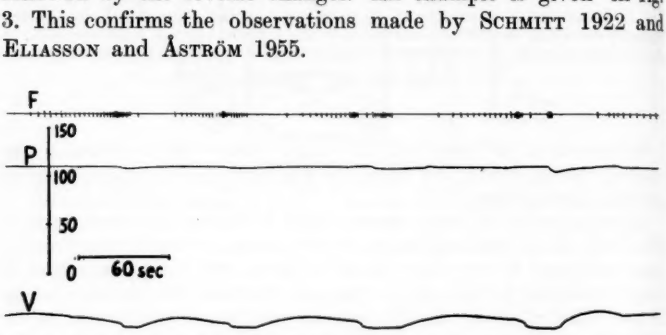

Fig. 3. Spontaneous activity recorded in flow (F), pressure (P) and volume (V). Placenta perfused under constant pressure.

III. Oxygen Consumption.

The oxygen saturation was measured in the blood before and after passage of different placentas. Constant output with a flow of 53 ml/min. was used. The blood was not diluted. Six samples were taken from the artery and six from the vein.

The arterial blood contained about 16 ml oxygen per 100 ml (range 15.83—17.80) and the venous blood about 13 ml oxygen per 100 ml (range 9.65—16.05). The placentas weighed 440—450 g. The mean consumption of the placenta was 3.7 ± 0.9 (E_M) ml oxygen per kg and minute.

IV. The Reaction of Placental Vessels to Oxygen.

1) *Constant perfusion pressure.*

The effect of different oxygen tensions to the fetal placental circulation was first studied with constant perfusion pressure. In these experiments the volume recorder was omitted. The

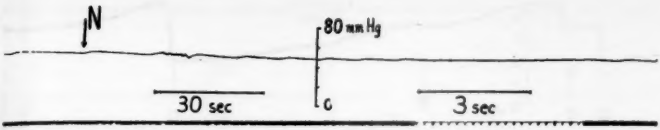

Fig. 4. The effect of hypoxemia to placenta perfused under constant pressure. Decrease of pressure and increase of flow from 8.6 ml/min. (oxygenated blood) to 16.5 ml/min. (hypoxemic blood). Arrow N indicates inflow of hypoxemic blood into the placenta.

placenta was kept in 37° Ringer-glucose solution. A change from oxygenated blood to hypoxemic caused a considerable increase of flow in 13 of 14 experiments and had no effect in one (Fig. 4). A change from hypoxemic to oxygenated blood made a distinct decrease of flow in all 15 experiments (Fig. 5).

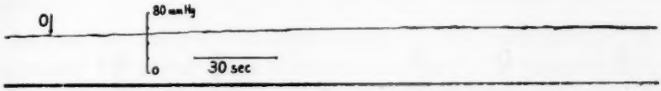

Fig. 5. The effect of oxygenated blood to placenta perfused under constant pressure Increase of pressure and a decrease of flow from 17 ml/min. (hypoxemic blood) to 5 ml/min. (oxygenated blood). Arrow O indicates inflow of oxygenated blood into the placenta.

2) *Constant output.*

A change from oxygenated to hypoxemic blood caused a decrease of the pressure in 14 experiments and had no effect in 4. A change from hypoxemic to oxygenated blood caused an increase of the pressure in 13 experiments and had no effect in 5 (Fig. 6).

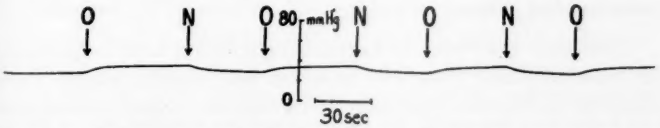

Fig. 6. Placenta perfused with constant output. Effect on pressure of different oxygen tensions in the perfused blood. O = blood with an oxygen tension of 222 ± 55 mm Hg. N = blood with oxygen tension of 17 ± 4 mm Hg.

3) *Isolated vessels* (circular muscle).

Placental artery. A change from oxygenated to hypoxemic blood caused shortening of the specimen in 13 experiments, lengthening in 16 and had no effect in 8 experiments. A change from

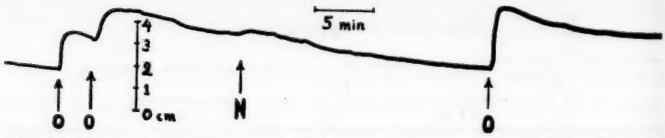

Fig. 7. Isolated placental artery. Effect on the circular musculature of different oxygen tensions. Abbreviations as in text to fig. 6. A deviation of 4 cm means a contraction of the specimen of about 20 %.

hypoxemic to oxygenated blood caused shortening in all 39 experiments (Fig. 7).

Placental vein. A change from oxygenated to hypoxemic blood lengthened the specimen in 7 experiments and had no effect in 3. A change from hypoxemic to oxygenated blood caused shortening in all 13 experiments (Fig. 8).

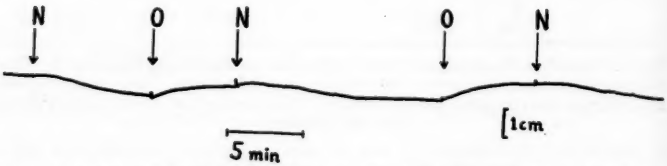

Fig. 8. Isolated placental vein. Effect on the circular musculature of different oxygen tensions. Abbreviations as in fig. 6 and deviations as in fig. 7.

V. Response to Drugs.

All the experiments on drugs were performed with constant pressure and volume recording.

Adrenaline was tested in 9 experiments. Every time a significant decrease in blood flow was recorded and simultaneously there occurred an increase in pressure and a decrease in placental volume.

Noradrenaline was tested in 12 experiments. In 10 of these experiments blood flow was significantly diminished, simultaneously the pressure increased and the placental volume decreased. The vasoconstriction induced by noradrenaline had a more or less rhythmic pattern. In two cases there were no significant differences.

Acetylcholine was tested in 5 experiments. In one experiment blood flow increased. In another the blood flow decreased and in the remainder there were no significant responses.

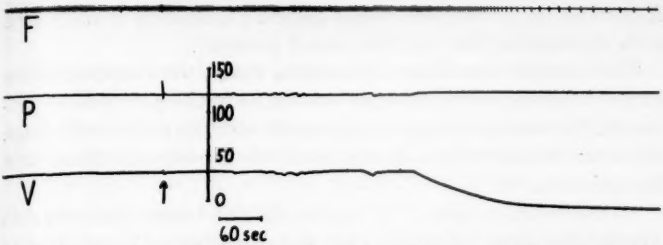

Fig. 9. Effect of vasopressin and oxytocin in combined preparation on placenta perfused under constant pressure. At arrow 0.1 IU of oxytocin and of vasopressin were injected into the afferent cannula.

Papaverine was tested in 4 experiments. Blood flow increased, but the changes in pressure and placental volume were not significant.

Oxytocin was tested in 6 experiments. In five cases blood flow decreased. In two of these experiments changes in pressure and volume were recorded. These changes had a rhythmic pattern.

Vasopressin and oxytocin, in combined preparation, was studied in 12 experiments. In all these experiments blood flow decreased and at the same time an increase in pressure and a decrease in placental volume were recorded (Fig. 9).

Discussion.

The oxygen consumption of the fetal part of the human placenta has not been estimated previously with heparinized human blood. The introduction of animals blood, blood of incompatible blood groups or blood containing citrate solution into the placental circulation may give rise to errors in the determinations of the oxygen consumption. In the present investigation, sodium citrate caused a pronounced contraction of the placental vessels. The present value 3.7 ± 0.9 ml oxygen consumption per kg and minute corresponds with determinations on resting skeletal muscle in living animals (HIMWICH and CASTLE 1927).

In the constant pressure experiments the reactions of the placenta to different oxygen tensions were easily reproduced. These results were confirmed in the experiments with constant output. An increase of the oxygen tension caused contraction of the placental vessels with reduced flow and increased pressure.

A decrease of the oxygen tension caused a dilatation of the vessels with accelerated flow and decreased pressure.

The circular musculature of isolated vessels were exposed to the same alterations of the oxygen tension as in the perfusion experiments. The reactions were in agreement with the previous findings. However, the responses of the artery to hypoxemic blood were inconsistent.

As indicated in table I the carbon dioxide tension was approximately the same in oxygenated and hypoxemic blood. It may therefore be concluded that only the differences in oxygen tension were responsible for the reactions. SCHMITT's investigations (1922) gave reason to the same conclusion.

This dependence of the fetal placental circulation on the oxygen tension of the fetal blood signifies a physiological purpose. Thus it can be presumed that the oxygen supply of the fetus is partly regulated by alterations in the fetal placental blood flow induced by changes in the oxygen tension of the blood.

It is of interest to compare the vascular reactions of the placenta with those of the lungs. NISELL (1951) found in perfusion experiments on cat lungs that a diminishing of the oxygen tension of the blood in the pulmonary artery decreased the vascular resistance probably by dilating the arteries and arterioles.

The drug tests completed previous investigations with the introduction of volume recording. Adrenaline and noradrenaline caused a constriction of the placental vessels and simultaneously a significant decrease of the placental volume was recorded. Acetylcholine and papaverine had no registerable effect on the placental volume but in other respects the results confirmed the observations made by V. EULER (1938) and ELIASSON and ÅSTRÖM (1955). The response to the combined preparation of vasopressin and oxytocin was a marked vasoconstriction and decrease of the placental volume. This is in agreement with the investigation of DAWSON and ROBSON (1940).

Similar results were obtained with the oxytocin preparation although the responses were less pronounced. DORNHORST and YOUNG (1952) found that oxytocin administered to the pregnant guinea pig had no effect on the fetal circulation. This might indicate that oxytocin did not pass the placental barrier, or that the responses of the fetal circulation were too small to be recorded.

Summary.

The response of human placentas to different oxygen tensions was studied in perfusion experiments using fresh human heparinized blood. Two different techniques of perfusion were used: constant pressure and constant output. The placental volume, the blood pressure near the afferent cannula and the flow rate through the placenta were recorded.

In other experiments the circular musculature of isolated placental vessels was studied. The alterations of the length of the specimen to different oxygen tensions in the blood were registered.

The tonus of the fetal placental vessels was found to be dependent on the oxygen tension of the blood. Vasoconstriction occurred when the tension was increased and vasodilatation when the oxygen tension was decreased.

The response of the fetal placental circulation to some drugs was studied by means of the constant pressure perfusion technique. These experiments confirmed the findings of previous investigators.

The oxygen consumption of the placenta was measured under constant flow. It was found to be 3.7 ± 0.9 ml O_2 per kg and minute.

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Solvent Drag on Non-electrolytes During Osmotic Flow Through Isolated Toad Skin and Its Response to Antidiuretic Hormone.

By

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It has been assumed, that if water penetrates a membrane passively, the fluxes of water from side I to side II of the membrane and from side II to side I are proportional to the water activities in the solutions bathing sides I and II respectively. However, in 1935 HEVESY, HOFER and KROGH found that the net water uptake by frogs sitting in tap water was 3 to 5 times higher than the value calculated from the influx of water measured with heavy water and the difference in osmotic pressure between frog lymph and tap water.

VISSCHER, FETCHER, CARR, GREGOR, BUSHEY and BARKER (1944) studied the water movement between gut and blood of the dog. In these experiments also the ratio between the fluxes into and away from the gut deviated from the ratio between the water activities in the blood and in the gut content, the net movement being in all cases too high. It was concluded that the water was subject to active transport across the intestinal wall.

Recently a possible explanation for the failure of the theory to fit with the experiments was suggested (USSING 1952) involving no active processes. The argument was that if water penetrates a membrane through pores an osmotic or hydrostatic pressure

difference across the membrane will cause a bulk flow of water through the pores (compare also JACOBS 1952). If so, molecules going in the direction of flow will be speeded up, whereas molecules moving in the opposite direction will be slowed down, thus making the ratio between influx and outflux different from the ratio of outside and inside activity of water.[1]

In a later paper (KOEFOED-JOHNSEN and USSING 1953) the pore theory was further developed. Some experiments, carried out on the water permeability of isolated anuran skins, confirmed the findings of HEVESY et al., and it was found that the neurohypophyseal hormone at least doubled the net water transfer through toad skin, whereas the diffusion rate of heavy water was only slightly increased (USSING 1950, KOEFOED-JOHNSEN and USSING 1953). The hormonal effect thus may consist in increasing the pore size in some layer of the skin without increasing the total area available for diffusion. On the other hand CAPRARO and BERNINI (1952) who also noticed this hormonal effect on isolated frog skin, believe that the water is actively transported across the skin.

If the pore theory has any reality one would expect to find solvent drag not only on water itself but also on solutes. Furthermore, the magnitude of the drag force should be related to the velocity of the solution in the pores. The aim of the present study was to test these consequences of the theory.

The general expression for the flux ratio of an uncharged substance is, according to the pore theory (see KOEFOED-JOHNSEN and USSING 1953)

$$\ln \frac{M_{in}}{M_{out}} = \ln \frac{a_o}{a_i} + \frac{\Delta_w}{D} \int_o^{x_o} \frac{2}{A} dx \quad \text{or} \quad \ln \frac{k_{in}}{k_{out}} = \frac{\Delta_w}{D} \int_o^{x_o} \frac{2}{A} dx \quad \ldots\ldots\ldots (1)$$

M_{in} and M_{out} designate influx and outflux, k_{in} and k_{out} the permeability coefficients, a_i and a_o the chemical activities of the substance in the inside and outside media, Δ_w net transfer of water, D the free diffusion coefficient of the substance, A the fraction of unit area available for diffusion, x_o the thickness of the membrane, and x the distance in the membrane from one

[1] If two mutually soluble substances pass through a membrane by dissolving in the membrane phase then there will be a small degree of interaction between them; however, the influence exerted by one substance upon the permeation of the other will be much less than when solvent drag is acting.

boundary.[1] This equation is only valid if the test substance does not cross the membrane partly by dissolving in the membrane phase, and if all pores are of the same shape and size.

If net water transfer and influx and outflux of a substance are measured simultaneously one should obtain a straight line when the logarithm of the flow ratio is plotted against net water transfer.

Thiourea and acetamide have been used as test substances because they can penetrate the skin and because they have very low lipoid solubilities. Each compound was used in two labelled forms. In the case of thiourea one form was tagged with S^{35} and the other with C^{14}, whereas acetamide was tagged with C^{14} either in the carbonyl group or in the methyl group. Further, heavy water has been used as a tracer for water.

A preliminary report on some of the experiments has been presented elsewhere (USSING and ANDERSEN, 1955).

Method.

The apparatus used was similar to that described by KOEFOED-JOHNSEN, LEVI and USSING (1952). By means of a spatula in each funnel to prevent splashing from the bursting air bubbles and siphons connecting the two stems of each funnel, the accuracy of the volume determinations was better than ± 5 μl. The ventral skin of the common toad (Bufo bufo) was used as a membrane. The exposed area was 7 cm^2.

After the skin had been mounted, 20 ml of each of the appropriate solutions were introduced into the inside and outside compartment, respectively, and the skin was allowed to equilibrate for 30 to 60 minutes. The two differently labelled forms of the test substance under study were now added, one form on each side of the skin. Thus one form served to measure the influx and the other the outflux. The concentration of test substance was kept sufficiently low (0.2—0.3 mg of thiourea per ml and about 2.5 mg of acetamide per ml) that no physiological effects could be observed. Usually the chemical concentration of the test substance was approximately the same on both sides. The system was allowed to equilibrate for another 30 minutes. Then duplicate samples were removed from each side and the volume of the outside solution measured. In the thiourea experiments the samples were replaced with the appropriate amounts of the original

[1] In the original paper the integral was erroneously given as

$$\int_{o}^{x_o} \frac{1}{A}\, dx$$

radioactive solutions. 2 to 4 hours later samples were again taken and the volume redetermined. 2 international units of mammalian posterior lobe hormone were added to the inside solution. Another 2 to 4 hours later samples were taken and the volume measured. Finally one sample from each side was removed and these were used as standards. After the experiment was finished the collected samples were analysed as described below.

In the cases when heavy water was used the experimental procedure was essentially that described by KOEFOED-JOHNSEN and USSING (1953).

The permeability coefficients of thiourea and acetamide were calculated according to the following formula:

$$\frac{a_2v_2 - a_1v_1}{a_sA\ (t_2 - t_1)}$$

Here a_1 and a_2 designate the radioactivity originating from side II in one ml of solution from side I at times t_1 and t_2, respectively, v_1 and v_2 the corresponding volumes of the solution on side I, A the area of the membrane and a_s the mean activity in one ml of solution from side II during the period from t_1 to t_2.

The formula used for the calculation of the water permeability coefficients was similar to that used by KOEFOED-JOHNSEN and USSING (1953).

Determination of heavy water. The water samples were purified according to the method described by SCHLOERB, FRIIS-HANSEN, EDELMAN, SHELDON and MOORE (1951) and the heavy water concentration determined by the gradient tube method (LINDERSTRØM-LANG, JACOBSEN and JOHANSEN 1938). The mean error of this method was about 3 per cent.

Determination of S^{35} and C^{14} in thiourea. The separation of the two radioisotopes was accomplished by oxidation of thiourea with bromine water in an acid medium. By this treatment thiourea was converted into sulphate and cyanamide. The sulphate was then precipitated with barium chloride. The cyanamide was converted to urea, split into ammonia and carbon dioxide and the latter was collected. The examples described below serve to illustrate the procedure.

The analysis for S^{35} was carried out in the following way. A 5 ml sample containing 1.41 mg of thiourea was acidified with hydrochloric acid and oxidized for at least two hours with an excess (0.5—1 ml) of bromine water. Next, carrier sulphate (22.7 mg potassium sulphate) was added and the sulphate precipitated as barium sulphate. The precipitate was thoroughly washed in order to remove every trace of cyanamide. Finally, for counting, the barium sulphate was transferred on to filterpaper which in turn was fixed on a brass holder (HENRIQUES, KRISTIAKOWSKY, MARGNETTI and SCHNEIDER 1946). The sample showed an activity of 75.9 counts/min. The weight of the barium sulphate was 34.6 mg, *i. e.* 11.0 mg/cm². The duplicate sample had an activity of 76.3 counts/min., and the weight was 10.9 mg/cm². The mean discrepancy between duplicate samples was 2 per cent.

For the determination of C^{14}, a 5 ml sample containing 0.99 mg of thiourea was acidified with sulphuric acid and oxidized with excess bromine water. After at least two hours the excess bromine was removed with ascorbic acid. The cyanamide formed was converted into urea by heating overnight at 50°. After adjusting the pH to about 3.5 the reaction mixture was transferred to the van Slyke gasometric apparatus and treated with urease dissolved in 0.5 M sec. potassium phosphate. After measuring the pressure of the carbon dioxide evolved (290 mm Hg, *i. e.* 60 per cent yield) the radioactivity of the gas was determined with an end-window G.-.M tube with a chamber attached underneath the window into which the carbon dioxide was transferred (USSING and ZERAHN, in preparation). When the activity was expressed in terms of 100 per cent yield, the counting rate was 44.3 counts/min. and for the duplicate sample 42.5 counts/min. The mean difference between duplicate samples was 3 per cent.

Determination of C^{14} in the carbonyl and methyl groups of acetamide. The principle used was the conversion of acetamide directly or indirectly to methylamine and carbon dioxide. If the reaction mixture is acid, methylamine is kept in solution while carbon dioxide is given off; the opposite is true in an alkaline medium. For the determination of C^{14} in the carbonyl group the Schmidt reaction (reaction of acetic acid with sodium azide to give methylamine and carbon dioxide) was used. For C^{14} in the methyl group the Hofmann reaction was used by which acetamide is also converted into methylamine and carbon dioxide. The Hofmann reaction could not be used for the determination of C^{14} in the carbonyl group because a small and variable part of the methyl carbon atoms are oxidized, whereas it is more convenient than the Schmidt reaction for determining C^{14} in the methyl group.

The Schmidt reaction was carried out in the following way. A 0.5 ml sample containing 1.25 mg of acetamide was pipetted into a small test tube (fig. 1). 10 μl of 50 per cent sodium hydroxide were added, and the acetamide hydrolyzed for one hour at approximately 75°. The test tube was then placed in a vacuum desiccator over solid sodium hydroxide. When the sample was dry 0.25 ml of purified kerosene and 0.1 ml of 100 per cent sulphuric acid were added. Any hydrochloric acid and carbon dioxide formed were removed by suction. About 25 mg of sodium azide were added and the test tube connected to the chamber of the van Slyke gasometric apparatus which contained 2 ml 0.5 N sodium hydroxide. To ensure a good yield of carbon dioxide the temperature of the reaction mixture was kept above 60°, by means of a little water bath, and the reaction allowed to proceed for not less than 20 minutes. Then the gas mixture was made to flow back and forth a few times between the reaction vessel and the chamber of the van Slyke apparatus by repeated decrease and increase of the pressure in the latter. By this procedure most of the carbon dioxide was absorbed by the sodium hydroxide. After acidification, the carbon dioxide pressure was determined in the usual way (141 mm Hg, *i. e.* 73 per cent yield). Finally, the carbon dioxide was transferred to the counting chamber mentioned above and the radioactivity measured. Expressed

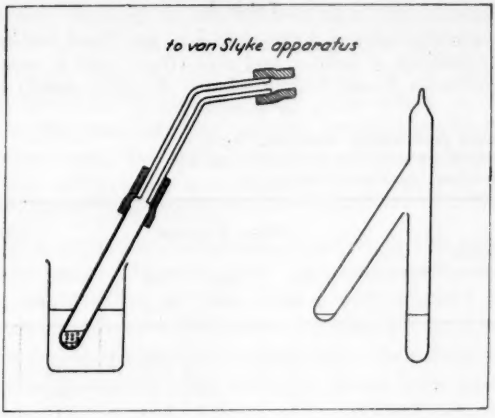

Fig. 1.

in terms of 100 per cent yield, the counting rate was 119 counts/min. for the sample and 124 for the duplicate. The mean difference between duplicate samples was 3 per cent.

The Hofmann reaction was carried out as follows. A 0.5 ml sample containing 1.25 mg of acetamide was placed in one arm of a λ shaped pyrex tube (fig. 1). In the other arm was placed sufficient sulphuric acid to absorb all the methylamine formed. The tube was cooled to 0° and 0.5 ml of a sodium hypobromite solution (20 μl bromine + 5 ml 20 per cent sodium hydroxide) added to the sample. The tube was kept for one hour at 0° before being evacuated and sealed. In order to liberate methylamine from the compound formed by the condensation of acetamide and sodium hypobromite the reaction mixture was heated overnight at 80°. The methylamine absorbed in the sulphuric acid was converted into carbon dioxide by the van Slyke—Folch wet carbon combustion (VAN SLYKE and FOLCH 1940, VAN SLYKE, PLAZIN and WEISIGER 1951). The carbon dioxide pressure as measured in the gasometric apparatus was 96 mm Hg, corresponding to a 50 per cent yield. Expressed in terms of 100 per cent yield, the counting rate was 194 counts/min. The activity of the duplicate was 204 counts/min. This method was slightly less satisfactory than the other analysis used, the mean discrepancy between duplicates being about 5 per cent.

Results.

The experimental data are presented in table I. When the solutions bathing the two sides of the membrane are full strength Ringer's the net water transfer is practically zero. Although the

Table I.

Effect of neurohypophyseal hormone (2 i. u. per 20 ml bathing solution) on the permeability of isolated toad skin (Bufo bufo) to water, thiourea and acetamide. Inside bathing solution Ringer's, outside solution as specified.

k_{in} = inward permeability coefficient (cm/h.)
k_{out} = outward permeability coefficient (cm/h.)
Δ_w = net water transfer (μl/cm^2/h.)

Date	Outside medium	Test substance	Before hormone				After hormone		
			k_{in} × 10^4	k_{out} × 10^4	$\frac{k_{in}}{k_{out}}$	Δ_w	k_{in} × 10^4	k_{out} × 10^4	$\frac{k_{in}}{k_{out}}$
26 March	Ringer's	Thiourea	6.63	4.72	1.40	0			
11 June			10.5	8.08	1.30	0	278	278	1.00
14 June			11.5	9.75	1.18	0	106	109	0.97
22 June			11.5	13.7	0.84	3	247	223	1.11
23 June			12.4	14.1	0.88	2	64.7	62.3	1.04
25 June			3.17	4.47	0.71	1	7.71	8.45	0.91
					1.05	*			0.98
15 June		Acetamide					537	516	1.04
22 June							363	321	1.13
25 March	1/10 Ringer's	Thiourea	2.90	3.03	0.96	8	20.6	16.2	1.27
6 April			6.48	6.51	1.00	14	17.4	14.4	1.21
8 April			11.4	8.73	1.33	14	49.3	31.2	1.58
26 April			4.00	4.02	1.00	8	7.52	6.42	1.17
29 April			4.25	4.07	1.04	17	15.4	12.3	1.25
3 May			9.86	6.52	1.52	14	156	113	1.38
25 May		Acetamide					251	121	2.08
1 June							175	105	1.67
6 June							299	180	1.66
10 June							530	239	2.22
29 June			129	100	1.29	24			
6 April			81.5	76.1	1.16	16	196	81.5	2.40
26 April		Heavy water	3,730	3,510	1.06	22	5,550	4,510	1.23
3 May			4,090	3,890	1.05	20	4,250	3,750	1,13
9 May			3,630	3,420	1.06	21	4,270	3,730	1.14
16 May			4,040	3,760	1.07	28	4,560	3,850	1.18

* Mean values. Value from 22 June "After hormone" excluded.

ratio k_{in}/k_{out} under these conditions may deviate from unity (especially when the permeability coefficients are small) the mean of the ratios is quite close to one. This demonstrates that neither before nor after the addition of posterior lobe hormone is thiourea subject to active transport across the toad skin. The same conclusion can be drawn concerning acetamide and water as men-

tioned below. Further, it can be seen that the permeability coefficients for acetamide and thiourea are increased by factors of from 2 to more than 20 times by the action of the hormone. The water permeability is also increased. The effect on net water transfer in the presence of an osmotic pressure gradient is much greater than upon the diffusion of water, in accordance with the findings of CAPRARO and BERNINI (1952) and of KOEFOED-JOHNSEN and USSING (1953).

There is a pronounced seasonal variation in the permeability of the toad skin. The extremely permeable state of the skin which is characteristic of toads during early summer lasts only for a few weeks. Since the thiourea experiments were completed before the period of excessive permeability, the results are biased in favour of acetamide and water as far as high permeability is concerned.

Discussion.

As the solvent drag is clearly evident, the toad skin must contain pores through which water and some solutes penetrate the skin. Furthermore, at least some pores must be common pathways for water and solutes. If all pores are accessible for both, the theory as expressed in eq. 1 requires that a plot of the logarithm of the ratio between the permeability coefficients (or the flux ratio) against net water transfer should give a straight line for every test substance.[1] As can be seen from fig. 2 this requirement is reasonably well fulfilled, the points representing acetamide and thiourea lying on the same line. The figure does not include points from thiourea and acetamide experiments without hormone because the accuracy was rather poor in these experiments. But a 'mean' of these data fits the line quite well. The points from water experiments without hormone from KOEFOED-JOHNSEN and USSING's paper (1953) fit the line for water very closely but are omitted for the sake of clarity. This may be taken as evidence for a nearly constant diffusion resistance to water, as already claimed by KOEFOED-JOHNSEN and USSING (1953). The fact that each line passes through the origin indicates that none of the substances is actively transported.

[1] Provided neurohypophyseal hormone does not alter the relation between rate of volume flow to rate of linear flow, *i. e.* only if the total area available for diffusion is unaffected by the hormone, should data from experiments with and without hormone fit the same line.

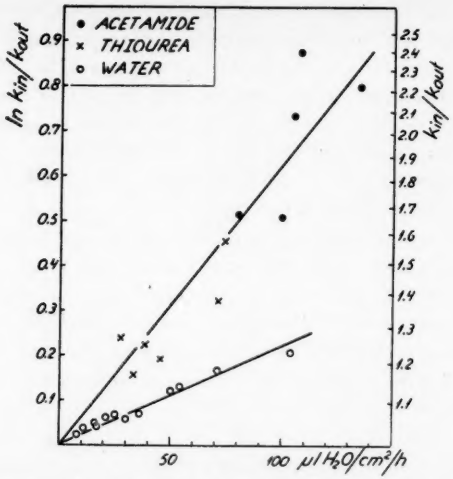

Fig. 2. The relation between ln k_{in}/k_{out} (which is proportional to the solvent drag force) and osmotically induced net water transfer (μl/cm²/h.). The graph includes only data from experiments with neurohypophyseal hormone. Some of the water data are taken from KOEFOED-JOHNSEN and USSING, 1953.

It can be predicted from eq. 1 that the ratio between the slopes of the lines must be equal to the ratio of the free diffusion coefficients. The values used for the diffusion coefficients are 2.4×10^{-5} cm²/sec. for water as measured with heavy water (WANG, ROBINSON and EDELMAN 1953), 1.0×10^{-5}. for acetamide (Int. Crit. Tables, *V*, 70) and 0.9×10^{-5}. for thiourea (calculated from the molecular weight and the diffusion coefficient of acetamide). The ratio between the slopes of the lines in fig. 2 is 2.8 as compared with the predicted ratios 2.4 and 2.7. A better fit between the theoretical and experimental values could not be expected, partly because of the scatter of the experimental data and partly because of the uncertainty of the values for the diffusion coefficients (see JOHNSON and BABB 1956). The results are consistent with the assumption that all pores are of (nearly) the same shape and size.

From eqs. 19 and 21 (KOEFOED-JOHNSEN and USSING 1953) it is possible to calculate the pore radius in an equivalent isoporous membrane with cylindrical pores. If the maximum flux ratio for water reported here and the minimum value from KOEFOED-JOHNSEN and USSING's paper (1953) are used for the calculation

one gets as maximum and minimum values for the pore radius 20 A and 6 A, respectively. The molecular radius of a water molecule is about 2 A (RENKIN 1954). Acetamide and thiourea probably have about the same radius as urea, namely 2.7 A. From these figures one would expect only a minor degree of molecular sieving (see for instance RENKIN 1954). However the concentration of acetamide or thiourea in the solution entering the inside solution is 20 per cent at most of the concentration in the outside medium, as can be seen from the values for k_{in}, k_{out}, and Δ_w.

This discrepancy between theory and experiments must mean that there cannot be continuous uniform pores, but that there is at least one obstacle which is passed more readily by water than by solutes. Such an obstacle may exist either as a homogeneous membrane or as a membrane with holes so small that, although water can pass without dissolving in the membrane matrix, acetamide and thiourea cannot. It seems likely that two membranes are present since, even in the high net water flow cases, the resistance to solutes is still high. Among other possibilities the following view is consistent with the experimental findings.

The main resistance to penetration lies in the two (outer and basal) boundaries of the epithelial cells. The outer boundary consists of a homogeneous membrane in which water is more soluble than are solutes. The properties of this membrane are not altered by neurohypophyseal hormone. The basal membrane is porous. Under the action of the hormone this membrane is changed in such a way, that although the individual holes are increased in size the total area available to diffusion is nearly constant. This model seems at the present time to be the most simple one consistent with the experiments but it is purely speculative and needs experimental support.

With certain assumptions, it is possible to calculate the flux value (M) for thiourea or acetamide in either direction in the case of no net water flow from the fluxes determined in an experiment with a net transfer of water. If it is assumed that the net permeation of thiourea or acetamide is governed by an expression analogous to Ohm's law, *i. e.*

$$RT \ln \frac{M_{in}}{M_{out}} = (M_{in} - M_{out}) R,$$

R for unit area having the dimensions of resistance, an approach analogous to that used by KOEFOED-JOHNSEN and USSING for the determination of water flux can be used. The equation for M becomes

$$M = \frac{M_{in} - M_{out}}{\ln (M_{in}/M_{out})} \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Permeability coefficients (k) may as well be used instead of fluxes. Calculated in this way k turns out to be almost identical with $1/2\ (k_{in} + k_{out})$. It must be pointed out that eq. 2 cannot be expected to have general validity, in contrast with the equation derived for the water flux.

Summary.

1. It is shown that the drag force exerted upon water and solutes by water is proportional to the osmotically induced rate of net water transfer across the toad skin.

2. The ratio between the drag effects on two substances is equal to the inverse ratio between their respective free diffusion coefficients.

3. These findings support the assumption that the toad skin contains a porous layer.

4. Neurohypophyseal hormone increases the permeability to both solutes and water, presumably by increasing the pore size in the porous layer.

5. It seems likely that in addition to the porous layer there is a homogeneous layer through which water can pass more readily than can hydrophilic solutes.

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Motor Hyperactivity Resulting in Diameter Decrease of Peripheral Nerves.[1]

By

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The way in which activity influences the fibre diameter of peripheral nerves in motor neurons is not altogether clear. It has been claimed that increased activity produces hypertrophy of nerve fibres (AGDUHR 1920, WEDELES 1949, EDDS 1950) and of nerve cell nuclei (WENDT 1951), and that loss of activity results in a decrease in the diameter of peripheral nerves (SZENTHÁGOTHAI and RAJKOVITS 1956). It has, however, been found in other cases that the diameter and development of motor nerve fibres are unaffected when their muscles are deprived of function (AITKEN, SHARMAN and YOUNG 1947, EVANS and VIZOSO 1951, EVANS 1953).

It will be shown in the present paper that motor nerve fibres may also react to hyperactivity by a decrease in diameter.

Experimental.

Animals. Young guinea-pigs reared in cages were used for the experiments. At the time of sacrificing, their weight ranged from 340 to 410 g. Six animals were used as controls and 15 for two series of experiments. They were killed by exsanguination through the neck vessels after unconsciousness had been produced by a blow on the head.

[1] The investigation was supported by a grant from AB Astra, Södertälje, Sweden.

Induction of increased motor activity. The animals were trained to run on an endless band at a rate of 0.8 m/sec. The running times were successively increased. One group of 8 animals was trained for about 14 hours on 11 consecutive days, and another group of 7 animals for 32 hours on 29 consecutive days. They were trained according to the following scheme:

Group 1	*Group 2*
1 × 10 min. for 1 day	2 × 10 min. for 12 days
6 × 10 min. for 3 days	2 × 15 min. for 5 days
4 × 10 min. for 1 day	3 × 30 min. for 2 days
6 × 15 min. for 3 days	3 × 45 min. for 10 days
6 × 20 min. for 3 days	

It proved necessary to give the animals a few minutes' rest in the middle of each period. The figures represent the actual running times.

Histologic technique. Two nerves from the brachial plexus on both sides were used, one supplying the serratus anterior muscle (n. thoracicus longus) and the other supplying the latissimus dorsi muscle (n. thoracodorsalis). In every instance, 1-cm long pieces at equal distances from the centre were taken.

The nerve samples were pinned on cork discs, after which they were stained with the Alzheimer—Mann—Häggqvist technique as described by REXED (1944).

The diameters of the nerve fibres, including the axons and myelin sheaths, were measured directly on photomicrographs and converted into fibre spectra. Altogether 6,823 fibres were measured.

Results.

In both experimental groups a decrease in fibre diameter was recorded, manifested as a shift to the left in the fibre size spectra (Fig. 1, *a* and *b*). This change was noted both in the bimodal nerve to the serratus anterior muscle, and in the unimodal nerve to the latissimus dorsi muscle.

For the nerve to the serratus anterior muscle, the mean value of the medians decreased from 4.58 μ to 4.31 μ in group 1, and from 4.58 μ to 4.16 μ in group 2, *i. e.*, by 6 and 9 per cent, respectively. The corresponding decrease for the nerve to the latissimus dorsi muscle was from 5.21 μ to 4.93 μ in group 1, and from 5.21 μ to 4.74 μ in group 2, *i. e.*, 5 and 9 per cent, respectively. The P value for both experimental groups as compared to the controls was less than 0.003 in the first case, and less than 0.015 in the second. No statistically significant difference was present between group 1 and group 2.

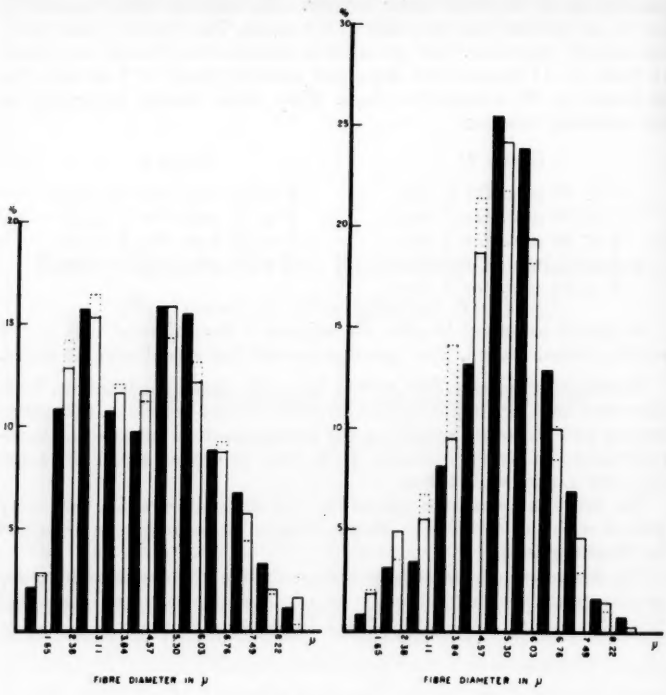

Fig. 1. *Fibre size spectra of the nerve innervating the anterior serratus muscle (a) and the dorsi latissimus muscle (b).* White staples denote the experimental animals, and black staples the controls. ——— = group 1, - - - - = group 2.

Discussion.

The present results with respect to the effect of hyperactivity on the dimensions of peripheral motor fibres differ from those obtained by other workers. Earlier reports as well fall into two apparently contradictory categories. Thus, some investigations indicate a positive influence of activity on fibre diameter (AGDUHR, WEDELES, EDDS) and on nerve cell nuclei (WENDT), whereas others seem to show that the degree of activity is of no importance for the size or development of motor nerve fibres (AITKEN, SHARMAN and YOUNG, EVANS and VIZOSO, EVANS). In order to evaluate the present observations of a decrease in fibre diameter on hyper-

activity, it is of interest to elucidate a mechanism which makes these results compatible with each other.

WEDELES, EDDS and WENDT all induced hyperactivity by the elimination of synergistically acting units. It is evident that such a procedure is apt to give rise to degeneration in the central nervous system. LIU and CHAMBERS (1956) have reported that collateral sprouting occurs in the spinal cord after denervation, and probably leads to formation of new synaptic end bulbs terminating on intact motor cell bodies and their dendrites. It has, however, been shown by BODIAN (1942 a and b) and by LEVI-MONTALCINI (1949) that the number of synaptic end bulbs is of importance for the dimensions of the neurons to which they become attached. Consequently, in these three cases it cannot be ruled out that the primary cause of the hypertrophy found was the formation of new synapses after central collateral sprouting. On the other hand, the results of SZENTHÁGOTHAI and RAJKOVITS, in which skeletectomy produced fibre atrophy, may have been due to loss of synapses, on account of unavoidable elimination of sensory fibres. AGDUHR's findings cannot be explained by either of these mechanisms. It is, however, conceivable that the system studied in this case differed fundamentally from the others referred to. This is because the hyperplasia observed indicates that the nervous system in question was immature.

If the mechanism outlined here did actually operate, the possibility exists that activity in itself has no direct action on motor fibre dimensions in the mature nervous system, which agrees with the findings of AITKEN, SHARMAN and YOUNG, of EVANS and VIZOSO, and of EVANS. In these cases, inactivity was produced by tenotomy, which is an intervention scarcely apt to cause any major damage, either to afferent or efferent nerves, resulting in central degeneration. Consequently, in these cases, "pure" activity or, rather, loss of activity, can be anticipated.

It may seem difficult to fit the present results as well into a general scheme. Investigations have, however, been made that seem to offer a plausible explanation. Thus, COOK and GERARD (1931) and ABRAMS and GERARD (1933) found that a nerve separated from its cell body lost its ability to conduct sooner if stimulated than at rest, indicating that substances necessary for function of the fibre are used up on activity, and that these substances are supplied by the cell body. HYDÉN (1943) found signs of intensified synthesis of proteins in the cell body as a

result of hyperactivity. GRANDE and RICHTER (1952) reported an increased rate of peripheral transport of ^{32}P compounds on electric stimulation of sciatic nerves in the frog. An increased consumption on hyperactivity is evidently met by an increased rate of synthesis in the nerve cell body, to ensure integrity of the functional mechanisms in the processes. There seems to be only slight support for the hypothesis that activity in itself has a dimension-increasing effect, with the possible exception of an acute one. Consequently, it seems probable that increased consumption alone is responsible for the increased synthesis of substance on hyperactivity. The possibility then exists that production may lag behind consumption on intense activity, whereas with more moderate variations in activity the demands are fully met by the regulating capacity of the cell body. In the former situation fibre atrophy is a likely finding, whereas in the latter no effect on the fibre dimensions is to be expected. In the present case, undue stress on the synthesizing mechanisms seems probable. Support for this assumption is afforded by a separate study made on the effect on the cell bodies of the hyperactivity in question. It showed the nucleolar volume in group 2 to have increased to 241 per cent of the basic value.

Summary.

Two groups of guinea-pigs were subjected to intense motor training. Fibre spectra of one unimodal and one bimodal nerve were recorded after training periods of 11 and 29 days, respectively.

The data recorded show that a significant decrease in fibre diameter can be produced by hyperactivity.

The mechanisms underlying the present findings are discussed. It is suggested that the consumption of substances in the processes may be so great on intense activity that it cannot be completely compensated by synthesis, thus resulting in a decrease in fibre diameter.

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Twitch Contractions of Isolated Flight Muscle of Locusts.

By

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The large longitudinal muscle in the wing-bearing segments of locusts and other flying insects is an indirect wing muscle which turns the wing downwards. In the desert locust, *Schistocerca gregaria*, this seems to be its sole function. EWER and RIPLEY (1953) concluded that the natural activity consists in prolonged series of non-fusing twitches. The present results are in accordance with their view and we have found no trace of a myogenic rhythm similar to the contraction rhythm which the homologous muscle displays in Hymenoptera and Diptera (PRINGLE 1949, ROEDER 1951). Since it can be assumed that the so-called fibrillar flight muscles have evolved from "ordinary" flight muscle, an analysis of the mechanical properties of the locust muscle may throw light upon the nature of specialization involved in the more advanced type.

The twitch properties of striated frog muscle are better known than those of other types, and the analysis of the present results has therefore been made in such a way as to make possible a direct comparison between frog muscle and locust flight muscle. This comparison was facilitated by the fact that the flight muscle resembles the frog muscle as far as structure, tetanic force and tetanic shortening are concerned (WEIS-FOGH 1956 a).

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Materials and Methods.

Material and preparation. Full grown, adult females of cage-bred desert locusts, *Schistocerca gregaria*, were provided by the *Anti-Locust Research Centre*, London. The morphology and the dimensions of the *dorsal longitudinal flight muscle* of the hindwings are described elsewhere together with the method of isolating the muscle (BUCHTHAL and WEIS-FOGH 1956). In order to prevent injury due to lack of oxygen and excessive shortening, preparation was done at 5° C and the muscle remained slightly stretched during the entire procedure apart from the short period of transfer from the preparation dish to the myograph. The muscle consists of parallel, prismatic fibres of approximately the same length; at each end they are attached to a strong piece of cuticle, the phragma, orientated perpendicular to the fibres. Each phragma was firmly fixed to a suspension clamp (Fig. 1 B) consisting of a piece of plastic with two vertical hooks (0.15 mm diameter, stainless steel). The tip of the hooks was fixed by means of a nickel-plated clamp, and small slabs of celluloid were wedged in between the plastic piece and the phragma, so that slack was prevented.

Saline, oxygen and temperature. It was difficult to keep the muscles excitable for more than one hour at 20° C and for more than 15 min. at 30° C, especially during tetanic experiments. The flight muscle is very sensitive to lack of oxygen (WEIS-FOGH 1956 a). Most of the results presented here therefore derive from twitch experiments at 10 to 11° C. At this temperature constant results are obtained for several hours.

The saline contained 175 mM Na^+, 5 mM K^+, 2 mM Ca^{++}, 161 mM Cl^-, 10 mM PO_4^{---}, 8 mM HCO_3^- and 0.25 per cent glucose. It was saturated with 5 per cent CO_2 in air so that the pH was 6.8 to 6.9. If saturated with oxygen instead of air, nitrogen disappears from the tracheae and causes them to collapse, impairing the respiratory exchange. The composition of locust blood is little known, but since the size and the duration of the action potential (internal electrode) was nearly the same in the freshly bisected animal and after it had been submerged in saline, the above saline hardly influenced the mechanical response compared with the response in the animal.

Stimulation. The muscle was stimulated with supramaximal single pulses of 5 msec duration from a square-wave stimulator *via* the multi-electrodes (Fig. 1B).

Myograph (Fig. 1). The isotonic myograph was of a new design. Force and shortening could be recorded in the same muscle either simultaneously or in quick succession. The muscle was suspended horizontally in saline with the one end firmly attached to the nearly static blade of the condenser of a condenser myograph (BUCHTHAL 1942) and with the moving end attached to a small piston which slides inside a polished glass tube. The movement of the piston was recorded photo-electrically. The tube was connected to a suction tank with constant pressure so that the piston and the muscle were loaded

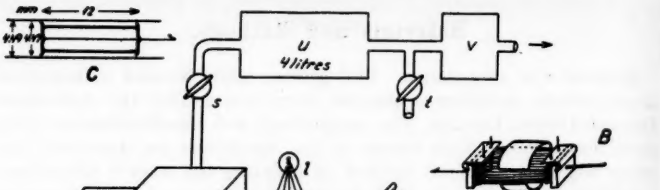

Fig. 1. Force and length recorder:
A. Vacuum myograph for isotonic and afterload recording (left) and condenser myograph for isometric recording (right).
B. Muscle mounted between suspension clamps and multi-electrodes for stimulation.
C. Piston of vacuum myograph. (Further details see text.)

by a constant force proportional to the difference in pressure between the atmosphere and the inside of the tank, the friction between the aluminium piston and the glass being small.

Fig. 1A shows the actual arrangement. The force recorder (a) and the muscle chamber (b) were placed on a common carriage (c) the position of which was controlled by the micrometer caliper (d). The muscle (e) was suspended between the piston (g) and the forceps (f) fastened to the condenser. It was submerged in saline the temperature of which was kept constant and read with an accuracy of $\pm 0.2°$ C (thermistor). 5 per cent CO_2 in air was bubbled through the saline. The wire (h) was of stainless steel, 60 mm long and 0.15 mm diameter, and passed through a slit in the muscle chamber. It was provided with three sharp, hook-shaped joints, one at the muscle clamp, the next at the gallow (i) and the third where the wire was fixed to the piston. The gallow (i) consisted of a thin, flexible thread of nylon (0.03 mm) which centred the piston in the tube. A small silver bead on the wire stopped the sliding steel disc (k; 0.15 mm thick) which was pressed against a fixed stop when the load exceeded the passive stretching load in isometric and afterload contractions.

The *Veridia* glass tube (m) had a specially polished circular bore, 4.49 mm in diameter, and the movements of the piston, or of its shadow, were recorded by means of an optical system consisting of the bulb (l; 6.5 V, 15 W), the two lenses (n_1 and n_2), the diaphragms (o_1 and o_2) and the photo-electric vacuum cell (p; Philips 90 AV). The change in voltage of the cell varied linearly with the movements of the piston and was recorded by the one beam of a double-beam cathode-ray oscilloscope (Dumont type 322). Stimulus and time markings were

traced by the other beam. Alterations in length were recorded on a continuously moving film with an accuracy of 0.01 mm, *i. e.* about 0.1 per cent of the muscle length.

The piston itself is shown in Fig. 1C and consisted of two pressed bottom pieces cut out of 0.05 mm aluminium foil and glued, by means of "Araldite", to a cylinder of 0.009 mm aluminium foil, 12 mm long. The diameter of the bottom pieces was 0.02 mm less than that of the bore of the tube (area 0.157 cm^2) so that the piston was nearly free of the wall in the rapid air stream. Moreover, the friction between glass and aluminium is low. When the piston was properly centred, and small oscillatory pressure changes were generated by a microphone in (q), the sinusoidal oscillations superimposed upon the tracing of the alterations in length did not show any significant distortion. The left hand side of the tube was connected with the tank (q; 0.9 litre), the pressure inside which was read on the inclined mercury manometer (r) and was reduced to the desired level by means of an electromagnetic piston pump via the container (v) and the two stopcocks (s, fine; t, gross). The tanks (u) and (v) served to damp the vibrations from the pump etc.

Accuracy. The length of the muscle in the body L_b was estimated by means of a travelling microscope with an accuracy of about 0.003 L_b. By means of the micrometer caliper (d), the alteration in length due to the changes in load or temperature were estimated with an accuracy of 0.005 L_b. During contraction, the stretching of the steel wire (h) was less than the accuracy with which the shortening could be recorded (0.001 to 0.002 L_b). The rate of change in length and force was estimated with an accuracy of about 5 per cent.

In this type of myograph, the actual load on the contracting muscle may be influenced in various ways. First, during shortening the air speed between the bottom pieces of the piston and the glass wall differs from the air speed when the piston is at rest. This altered the actual load during contraction, but in the most unfavourable case (lowest load and highest speed at 30° C) the average air speed changed insignificantly by only 0.5 per cent. The hydrodynamic resistance of the saline was also insignificant. During the fastest contractions Reynolds number varied from 50 to 200, the width of the muscle clamp being used as reference length. The resistance coefficient for the clamp would then hardly exceed 2 and the hydrodynamic drag hardly 2 mg, which is of no importance. A third factor was the inertia of the piston, the wire, and the moving muscle clamp. Their total mass corresponded to a weight of 45 mg. The maximum force recorded by the myograph was about 50 g and the apparatus therefore compares well with other myographs of high sensitivity (cf. ABBOTT and RITCHIE 1951; 75 mg weight to 40 g force). Since the *average* maximum acceleration approached 170, 1,000 and 4,200 $cm \cdot sec^{-2}$ at 10, 20 and 30° C respectively, half of the muscle weight of 20 mg added to that of the moving parts, produced corresponding extra forces due to acceleration of 10, 55 and 230 mg at the three temperatures. Although small, some distortion was likely to occur at the smallest load (1.2 g) at the highest temperature (30° C), but the forces were insignificant at 11°

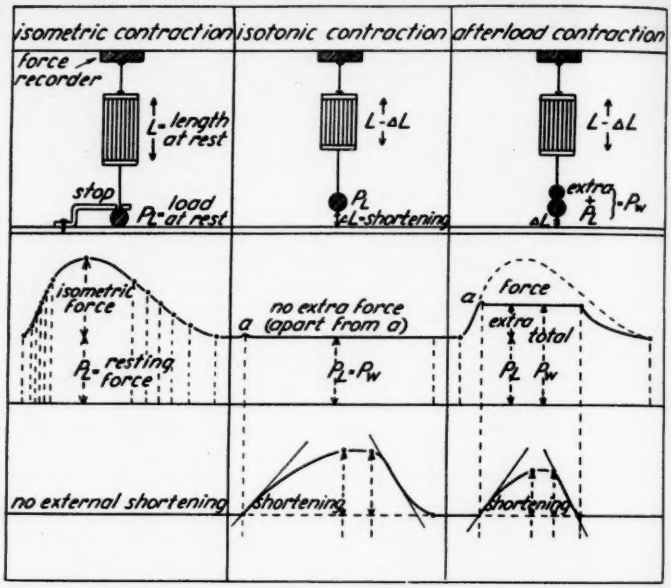

Fig. 2. The three types of contraction examined and the quantities measured. The force is symbolized by a weight P, but in the experiments P had practically no inertial mass.

and 20° C. Nevertheless, a transient increase in force was observed at the beginning of the shortening; it lasted 4 to 8 msec and amounted to about 0.5 to 1.0 g at 10° C and to about 1.0 g at 20° C. This hump (cf. *a* in Fig. 2, and Fig. 9) decreased when the subsequent shortening velocity decreased and model experiments indicated that it was caused by the combined effect of inertial force and friction between piston and glass. Its effect on the muscle was similar to a quick loading and unloading by 0.5 to 1 g at the beginning of the shortening. The duration of this artifact was identical with the initial delay in the onset of shortening mentioned on page 259.

Procedure.

The cage-bred locusts are very similar in relative proportions, muscle weight etc. (Weis-Fogh 1952). When the isolated resting muscle was stretched by a force $P_L = 6.0$ g its length equalled to within 0.1 mm the length L_b in the bisected thorax before preparation; it averaged 7.47 mm. In the experiments, P_L was altered in steps from 1.2 g (length = 0.91 L_b) where nearly all fibres were under tension, up to 18.1 g (length = 1.10 L_b). The stretching could not be

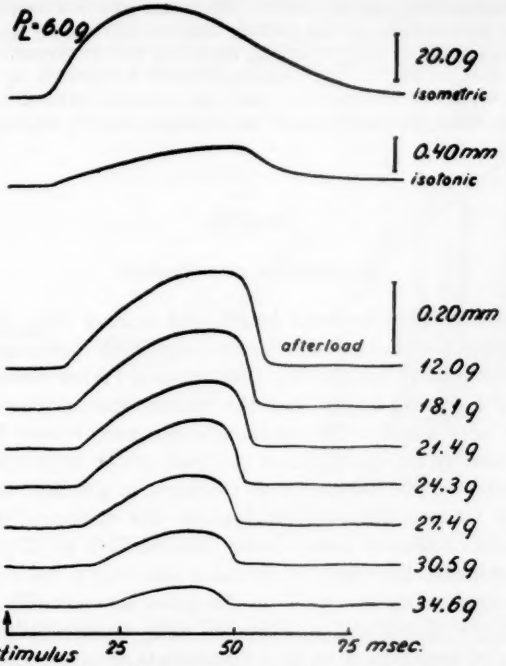

Fig. 3. A typical series of flight muscle contractions, starting from the length in the body (20.2° C). The different loads are indicated to the right hand side of the seven afterload curves.

increased any further because the total force exerted in an isometric twitch then exceeded 45 g so that lasting injury would be inflicted (WEIS-FOGH 1956 a). Compared with frog muscle the possible range of lengths is small; the flight muscle is less extensible and, in the locust, its length does not go beyond the above limits.

The procedure is illustrated in Fig. 2. At the beginning of a series of recordings, the muscle was loaded by P_L grams and given a few *isotonic* contractions where it shortened against P_L. Then the resting length (L) was estimated and the stop (k) in Fig. 1 was adjusted to this length. The load on the piston was increased to more than the total force exerted during a twitch (resting force + twitch force) so that the force could be recorded during an *isometric* twitch. At a series of intermediate loads P_w, the muscle contracted as in an isometric twitch until the total force reached P_w; then it started to shorten against this load. This type is called *afterload* contraction and both the alteration in length and in force could be recorded. Some typical tracings of force and length are shown in Fig. 2 together with the

quantities measured on the curves. The series was concluded with an isometric contraction; if the twitch tension differed by more than 10 per cent from the first recording, the series was discarded. A typical series is seen in Fig. 3. The technique made it possible to compare the three types of contraction. Since the muscles differed very little from each other, the results could be averaged directly for each length and load.

Results.

(a) Tetanus and Twitch.

The relationships between length and tension (Fig. 4) at rest and during contraction show two interesting features: (1) At 11° C, the tetanic force of the flight muscle (WEIS-FOGH 1956 a) was only 1.3 times higher than the twitch force; at this temperature the activation of the contractile component must therefore still be near to its maximum at the peak of the isometric twitch. The small difference between force produced in a twitch and force during a tetanus indicates furthermore the existence of a relatively non-compliant series elastic element (see p. 272). (2) On the other hand, the twitch shortening was only a fraction of the tetanic shortening, as seen from the ratios given in Fig. 4 (left); the relative shortening was about eight times smaller than in twitches of frog muscle at 0° C (BUCHTHAL, KAISER and ROSENFALCK 1951). This indicates that the relative decay of the active state is quicker in locust flight muscle than in the skeletal muscle of frog. A small load of 0.03 P_o (P_o = tetanic force) resulted in a twitch shortening of only 0.13 L_b (L_b = length in the body) while an increase in the load to 0.3 P_o reduced the twitch shortening to 0.03 or 0.04 L_b. In this respect the flight muscle is clearly specialized. It is important to note that the length of the twitching muscle remained close to the optimum for force production, 0.9 to 1.1 L_b (WEIS-FOGH 1956 a). In other words, the alteration in length during a twitch did not cause any appreciable alteration in the geometry of the contractile components, as it does in most other types of striated muscle. Since, however, the non-active flight muscle exerts considerable resistance against passive stretch (see "rest" curve in Fig. 4), the small degree of shortening may nevertheless cause an appreciable release of energy stored in passive elastic components. This is further dealt with in the following sections.

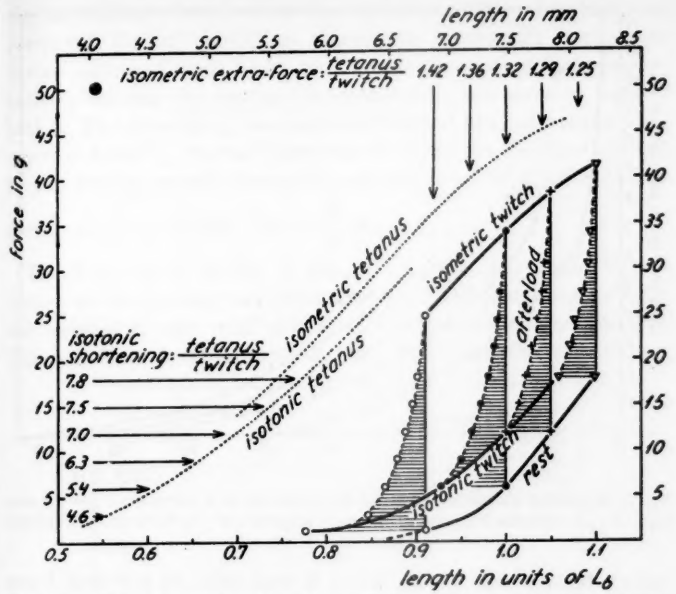

Fig. 4. Length-tension relationship of locust flight muscle at rest and at the peak of single twitches at 11° C (full curves) compared with the tetanic values (dotted curves, from WEIS-FOGH 1956 a). The horizontal hatching shows the extent of the twitch shortening. Average of nine muscles.

The tetanic force increases much with increasing temperature (WEIS-FOGH 1956 a) from 1.5 kg per cm² at 11° C to 3 kg per cm² at 25° C. Neither the twitch force nor the twitch shortening were affected to the same extent: the ratio between tetanic force and twitch force was about 1.1, 1.3 and 2 at 3°, 11° and 25° C respectively. Thus the effect of an increase in activation with temperature which manifests itself by the increase in tetanic force seems largely to counteract the more rapid decay of the active state associated with the higher temperature (RITCHIE 1954 a).

(b) Force, Shortening and Work in a Twitch.

Isometric force. Table 1 shows the peak values observed at different lengths and loads at 11° C. Maximum of contraction force was produced at the length in the body, L_b, where the

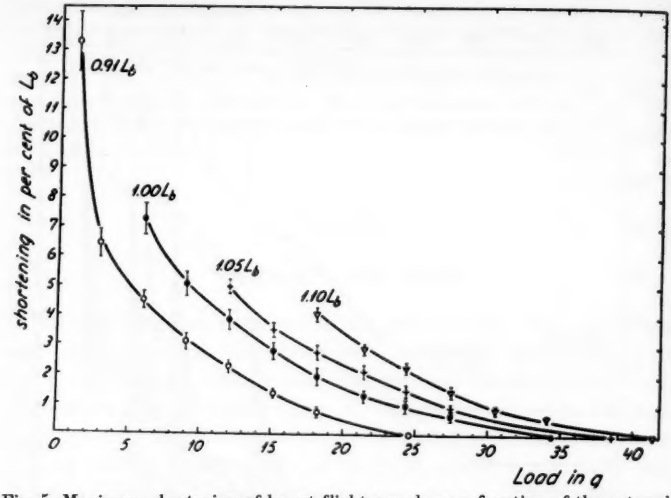

Fig. 5. Maximum shortening of locust flight muscle as a function of the external load (P_w) in twitches initiated at different lengths (11° C). Vertical bars indicate the mean error.

twitch tension was 1.4 kg · cm^{-2}; it was only 16 per cent lower at 1.10 L_b when the passive pull amounted to 1 kg · cm^{-2}. The twitch force increased by 1.2 times from 10° C to 20° C and further by 1.3 times from 20° C to 30° C so that the peak tension was about 2 kg · cm^{-2} at the lowest temperature for flight (30° C; WEIS-FOGH 1956 b). In frog muscle, on the contrary, the twitch tension is nearly halved when the temperature is increased from 0° to 20° C (HILL 1951).

Shortening. Fig. 5 shows the observed relationship between the twitch shortening and the total external load P_w against which the muscle worked.

The shortening against a given external load was the larger the more the muscle had been stretched at rest, although the length of the muscle, even during shortening, remained near to that which gives maximum tetanic force. The differences in the degree of shortening could therefore not be explained by the way in which P_o varies with the length of the flight muscle (Table 1). They might, however, be due to the increase in resting force with stretch, the work performed when stretching the muscle being stored as elastic energy and reconverted into mechanical work during the short period of shortening. To test this possibility

the shortenings initiated at the different resting lengths were compared during conditions where the contractile component carried identical loads by assuming that the resting force resided entirely outside the contractile component and were in parallel with it. The shortening was therefore plotted not against the total external force P_w, but as a function of P_w minus the force exerted by the resting muscle averaged over the range of lengths passed during a given twitch: $P_w - \frac{1}{2}(P_L + P'_L)$.

The four curves of Fig. 6 obtained in this way could be expected to be parallel and separated by *small* distances in some way related to the small differences in isometric force given in Table 1. This is approximately the case, but the shortenings

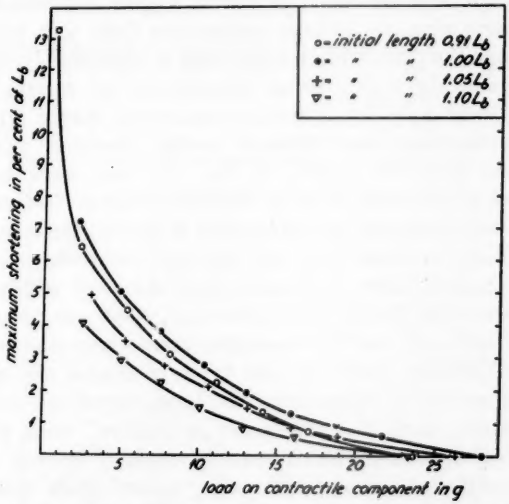

Fig. 6. Maximum twitch shortening as in Fig. 5 but, instead of as a function of the total load P_W, it is plotted as a function of $P_W - \frac{1}{2}(P_L + P'_L)$, where P_L = resting force at the initial length and P'_L = resting force at the shortest length during the twitch. This load is called "load on the contractile component".

given in Fig. 6 are somewhat smaller the more the resting muscle is stretched. It seems therefore reasonable to assume that the main part of the force, in fact, is localised outside the contractile component and performs mechanical work during the shortening in a twitch. This assumption is further analysed in the following section.

Table 1.

Isometric force in twitch at 11° C.

Length in units of L_b	Load P_L (g)	Isometric extra force (g)
0.91	1.2	23.6 ± 0.8
1.00	6.0	28.0 ± 1.3
1.05	12.0	26.3 ± 0.7
1.10	18.1	23.4 ± 1.2

Mechanical work. The product of the external load P_w and the maximal shortening ΔL is the total work performed by the muscle in a twitch. It is illustrated in Fig. 7A as a function of the external load for the four muscle lengths considered. Each curve starts with an isotonic contraction (left) and ends with an isometric one. It is seen that the work is considerably increased by stretching the muscle before stimulation, but a large part of the total work may derive from stored elastic energy ("passive" work) rather than from chemical energy liberated during the contraction ("active" work). In Fig. 7B, the work has been calculated as the work done by the contractile or "active" components assuming that the differences in stored elastic energy at length L and at length $L - \Delta L$ has been completely converted into mechanical work. It is seen that the large differences in work have disappeared and, moreover, that the relationship between work and load now resembles that of frog muscle (BUCHTHAL and KAISER 1949); in both types of muscle the optimum load is about 0.3 P_o. Considering the large corrections introduced for "passive" work the differences in "active" work produced at different degrees of stretch are only small: In isotonic contractions at different initial lengths the "active" work was almost identical in spite of a correction for passive work of as much as 86 per cent of the total work at length 1.1 L_b. The apparent decrease in "active work" with stretch might be due to an incomplete conversion of passively stored elastic energy. The curves of Fig. 7B indicates this loss to be 0.04 to 0.08 gcm at 11° C. This is small as compared with the 0.20 gcm "active work" performed at optimum load. The work performed by the "passive" component could amount to 0.40 to 0.50 gcm (at 1.10 L_b) and is clearly a factor of great mechanical importance in the flight muscle.

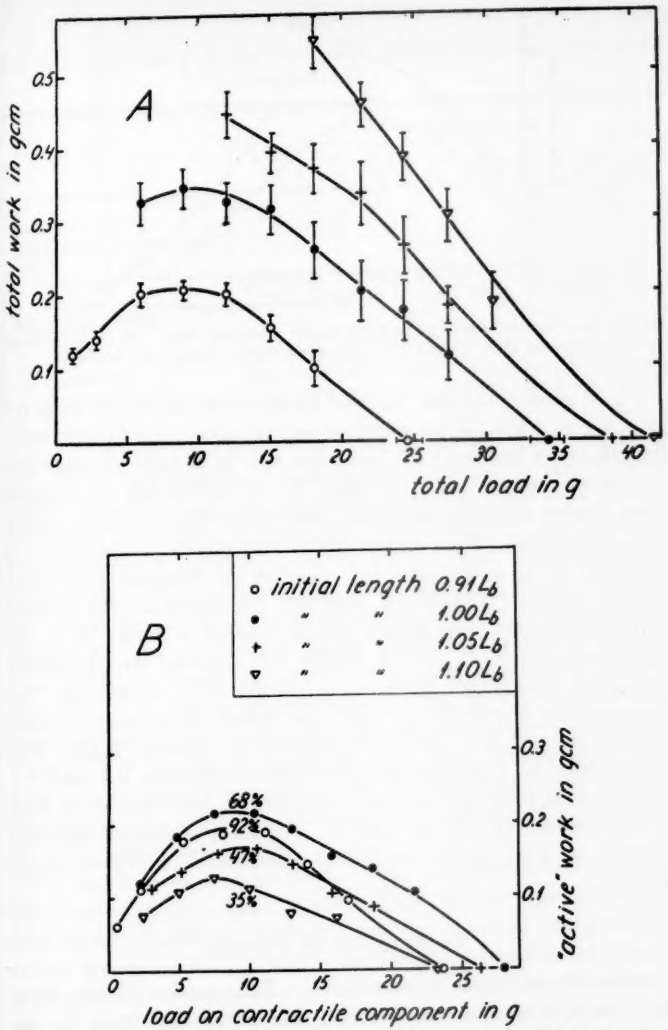

Fig. 7. A. *Total twitch work* of nine flight muscles as a function of the total external load P_w (11° C). The vertical bars indicate mean error. B. The reduced or "active" part of the total work as a function of the load on the contractile component. The twitch work was calculated as shortening times load on the "contractile component" (see Fig. 6; $\Delta L\,[P_w - \frac{1}{2}(P_L + P_L)]$. The percentages show how much of the total work is considered "active" at the optimum load of about 10 g.

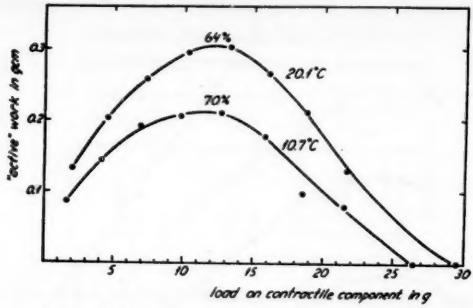

Fig. 8. The "active" part of the total twitch work (cf. Fig. 7 B) in three flight muscles at 10.7° C and 20.1° C, respectively. Length in the body.

Fig. 8 shows that the "active" twitch work increased from 0.2 to 0.3 gcm when the temperature was increased from 11° C to 20° C (length in the body); at 32° C, in the few successful experiments, the work approached 0.5 gcm. It is interesting to compare these figures with the metabolic rate of the flight muscles during sustained flight (WEIS-FOGH 1952); the muscle temperature is then 30° C or above and the wing stroke frequency is about 18 per second (WEIS-FOGH 1956 b). According to the present experiments, the mechanical power output ("active") of the twitching muscle of 11 mg weight can amount to 70 calories per gram muscle per hour as compared with an average fuel consumption of 400 $cal \cdot g^{-1} \cdot h^{-1}$; the latter figure may increase to 800 $cal \cdot g^{-1} \cdot h^{-1}$ during exceptionally vigorous flight. This corresponds to a mechanical efficiency of between 0.1 and 0.2. Independent estimates on the flying animal gave values between 0.14 and 0.18 (WEIS-FOGH 1956 c). The *isolated* muscle therefore seemed to work under conditions comparable to those in the animal.

(c) Duration of Twitch.

Duration and type of contraction. The passive elastic force acted as if it was in parallel with the contraction force. In isotonic contractions the passive elastic component should therefore experience the same deformation as that of the contractile component; the total duration of the *mechanical change* will then be determined by the component whose return to the resting state takes the longest time. Under isometric conditions, the parallel

Table 2.

Duration and latency of the mechanical change in twitch contractions at 11° C.

	Resting load			
	1.2 g	6.0 g	12.0 g	18.1 g
Total duration (from onset of stimulus) in msec				
isotonic	368 ± 24	314 ± 21	331 ± 26	362 ± 30
isometric	271 ± 16	318 ± 17	349 ± 15	346 ± 24
Mechanical latency in msec				
isotonic	24.2 ± 1.6	27.0 ± 1.6	29.3 ± 1.9	27.0 ± 1.4
isometric	20.4 ± 0.8	21.8 ± 0.5	21.3 ± 1.2	18.9 ± 0.8

elasticity remains the same and the contractile component alone determines the duration. A comparison between the isotonic twitch duration and the isometric duration does not show a significant difference between the two types (11° C, Table 2). The isometric value (271 msec) at 1.2 g of load is probably not relevant since part of the fibres were known to be slack before the contraction. The time of adjustment of the passive elastic component is therefore not longer than that of the contractile component; it is probably shorter. Actually, when the force was recorded during afterload contractions (Fig. 9), the total duration of the change equalled the isometric duration (upper and lower tracing), but the rapid decline in force, seen immediately after stretch, indicates the presence of small, rapid adjustments within the passive elastic system. Dynamic experiments are still lacking on this point, however.

The mechanical latency did not vary significantly in isometric experiments at 11° C (ca. 20 msec; Table 2); the small difference between the isometric and the isotonic readings are probably caused by the initial "hump" mentioned on page 250 (cf. Figs. 2 and 9). The action potential lasted about 15 msec at this temperature (measured with intracellular electrodes) and varied in the same way with temperature as the latency. The electrical event was therefore terminated before the onset of contraction.

Effect of temperature. The temperature dependence was studied in isometric twitches. The total duration of the

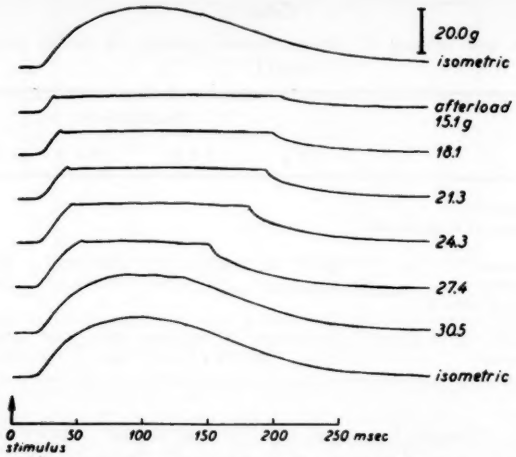

Fig. 9. Change in force during twitches of locust flight muscle with different afterloads, as indicated at the right hand side (initial length 1.05 L_b; 10.4° C). The series starts and ends with an isometric recording; note the small initial "hump" at the plateau of force.

mechanical change in Fig. 10 (logarithmic ordinate) decreased by a factor of 6 from 0° to 10° C, by 2.7 from 10° to 20° C, by 2.1 from 20° to 30° C, and by 1.6 times from 30° to 40° C. The diagram also shows that the ratio between the rising phase of the twitch and the total duration increases with temperature, *i. e.* the relaxation becomes relatively more quick; the same happens in frog muscle (BUCHTHAL, KAISER and ROSENFALCK 1951).

From a biological point of view it is interesting to compare the duration of the twitch with that of the natural wing stroke cycle (WEIS-FOGH 1956 b). In Fig. 10 the latter is shown by the horizontal arrow, the width of which corresponds to the normal variation due to differences in flight performance and size. The vertical arrow indicates the *lowest* muscle temperatures recorded during sustained flight, 30° to 32° C, the highest muscle temperature being 42° C. When the thoracic temperature is less than 30° C, the wing stroke frequency decreases and flight cannot be maintained, perhaps because of partial fusion of the twitches or because the metabolic recovery is too slow. Between 30° and 42° C, the duration of the wing stroke remains constant (about 58 msec) so that complete mechanical relaxation of the flight

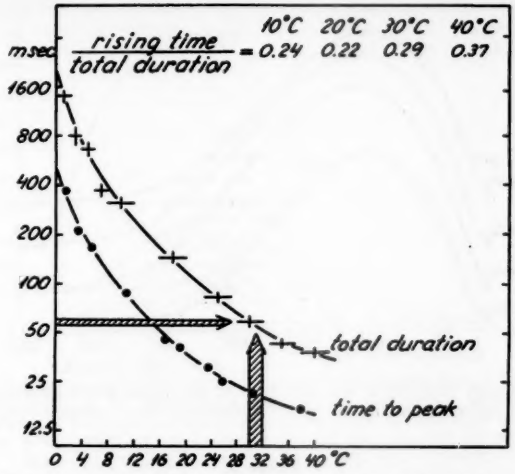

Fig. 10. Influence of temperature (abscissa) upon twitch duration and time to peak tension (logarithmic ordinate) in locust flight muscle. Vertical bars = mean error; horizontal bars = range of temperature. The vertical hatched arrow is the lowest temperature during sustained flight and the horizontal arrow is the duration of the natural wing stroke.

muscle is ensured. But Fig. 10 also shows that an increase in muscle temperature from 30° to 40° C reduces the twitch duration as well as the time to peak tension by about 1.5 times. This relationsship can therefore not explain why, in fact, the wing stroke frequency is independent of the thoracic temperature within this range (WEIS-FOGH 1956 b). Since the flight can go on for hours at unreduced rate and since the "oxygen debt" is quite negligible compared with the enormous amount of fuel combusted (KROGH and WEIS-FOGH 1951), the metabolic cycle of the twitching muscle must include complete recovery within the period of mechanical response. There can be no piling-up of intermediate metabolites.

(d) Variation of Force and Length with Time.

Isometric force. Fig. 11 shows the average isometric force as a function of time at four different lengths. The four curves are nearly identical; the vertical displacements correspond to the load P_L with which the resting muscle was stretched. Both the rate of rise and the rate of fall in tension were independent

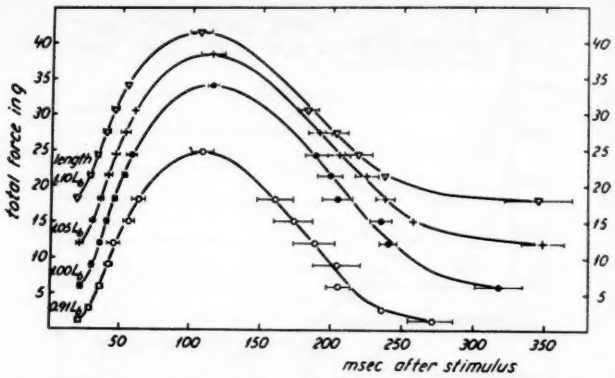

Fig. 11. Average variation in isometric force of nine flight muscles during twitches at different lengths at 11° C. Horizontal bars = mean errors.

Table 3.

Maximum rates of tension rise and fall (11° C).

Length in units of L_b	Resting load in g	Rate of rise in tension in kg cm^{-2} sec^{-1}	Rate of fall in tension in kg cm^{-2} sec^{-1}
0.91	1.2	30 ± 3	12 ± 2
1.00	6.0	36 ± 3	10 ± 1
1.05	12.0	36 ± 3	12 ± 1
1.10	18.1	37 ± 3	14 ± 1
		average 35	12

of the stretching force (Table 3), as they should be when the passive elastic force acts in parallel with the contraction force. The rate of tension rise reached its peak about 4 msec after the latency period and remained nearly constant until 50 msec after the stimulus, *i. e.* during 25 msec or 7 to 8 per cent of the total twitch. If at all present, the "plateau" of full activity was brief, as is also the case in frog muscle (MACPHERSON and WILKIE 1954). At 11° C the tension increased by 35 $kg \cdot cm^{-2} \cdot sec^{-1}$, or by 20 $P_o \cdot sec^{-1}$, where P_o is the tetanic force at this temperature. At 20° C the rates of rise and fall had increased by 3.3 times but since P_o also increased, the maximum rate of rise in tension was about 40 $P_o \cdot sec^{-1}$. Exact figures were not available for frog

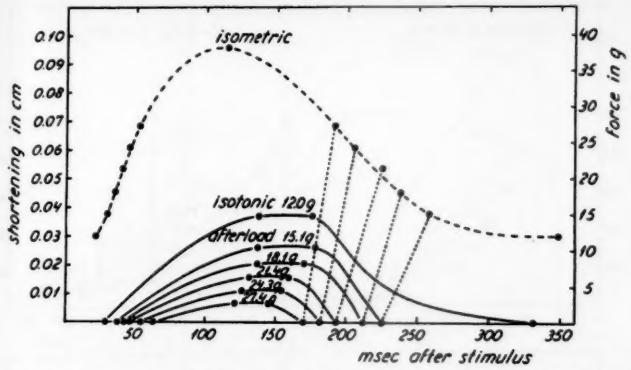

Fig. 12. Average course of shortening of nine flight muscles during isotonic and afterload twitches at 11° C, resting length 1.05 L_b. The upper, broken curve is the variation of the isometric force and the straight, dotted lines connect the end-points of the afterload curves with points on the isometric curve where the instantaneous force equalled the afterload in question.

muscle but seem to be of the same order of magnitude (MACPHERSON and WILKIE 1954).

Shortening and stretching. Fig. 3 is a photographic recording of a typical contraction series and Fig. 12 gives the average values from nine muscles at 11° C, the resting length being 1.05 L_b. The general shape was the same at other loads and temperatures. The shortening velocity is treated separately but a few other features need comment. The shortening phase ended with a rather prolonged stand-still lasting for about one tenth of the twitch period; then the stretching phase began and, in all the *afterload* experiments, the elongation occurred two to four times faster than the shortening. Stretch ceased when the force which the muscle was just able to carry during elongation had fallen to the afterload value P_w. It is now interesting to compare this instant of time with the time at which the isometric force had fallen to P_w. If the activation of the contractile units is not affected by the type of contraction and if the time of adjustment of the passive elastic components is small, the two events should coincide in time. The straight, dotted lines in Fig. 12 show that the elongation was completed a little before it should be expected according to the isometric curve. This "hastening" of the relaxation was larger the larger the preceding shortening had been but the measurements do not allow any detailed analysis; it averaged

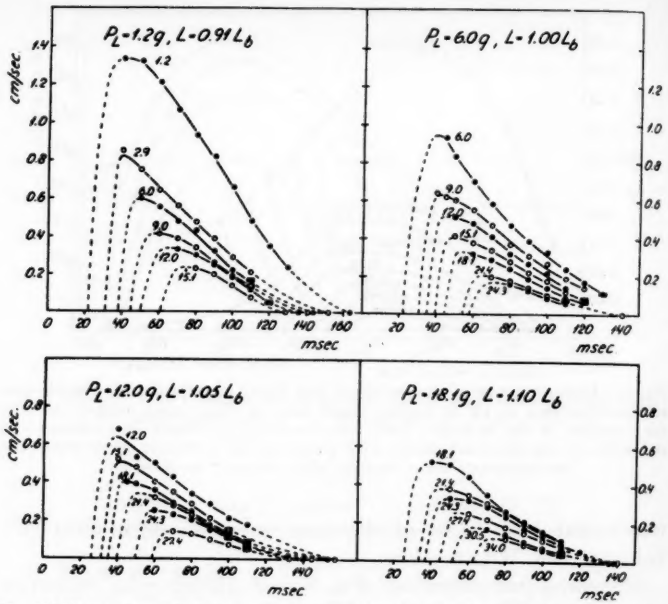

Fig. 13. Shortening velocity as a function of time in isotonic and afterload twitches of locust flight muscle initiated at four different lengths (averaged from six muscles, 11° C). The afterload (P_W) is indicated on each curve.

12 msec. At the time in question, 12 msec on the isometric curve corresponded to a difference in force of 2 or 3 g, or to 10 per cent of the isometric twitch force. The force which the whole muscle can carry is therefore affected to only a small extent by the type of contraction; the observed differences may be caused by the dynamic properties of the passive elastic system (nature of damping) or, perhaps, by a *small* "deactivation by quick release" (PRINGLE 1954).

Shortening velocity and power. Under all conditions investigated here, it was characteristic that there was no interval of time during which the shortening velocity remained constant. After the initial period of acceleration, lasting less than 10 msec at 11° C, the velocity reached a maximum and started to decline. From then on, it decreased about linearly with time until the shortening was over. The curves in Fig. 13 are average values from six muscles at 11° C; the velocity was estimated by numerical

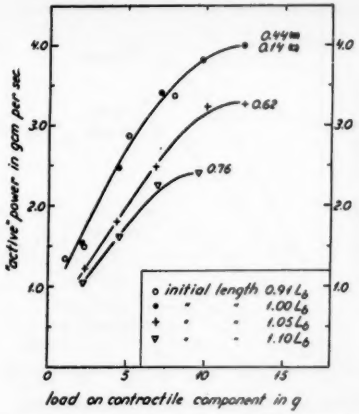

Fig. 14. The reduced or "active" power of locust flight muscle 60 msec after the stimulus, calculated as shortening velocity times $P_W - P'_L$ (= "load on contractile component"), where P'_L is the passive elastic force of the resting muscle at the length which it had 60 msec after stimulation. This length differed according to the afterload and the four initial lengths considered. The fractions at the upper end of the curve show how much of the total power is estimated to be due to passive elastic forces.

differentiation of the photographic recordings. When half the shortening was completed, the velocity had decreased by 15 per cent and when 0.8 of the shortening was completed, the velocity was half its initial value.

At a given instant of time the velocity multiplied by the external load P_w is the *power* of the total muscle; it does not represent the power of the contractile components because the passive elastic components carry some of the load. This part of the load is unknown but it cannot exceed the pull P'_L which the resting muscle exerts at the length to which the active muscle has shortened at that instant. P'_L can be estimated from the static length-tension diagram in Fig. 4. If, therefore, the instantaneous shortening velocity is multiplied by $P_w - P'_L$, we get the lower limit for the power of the active, contractile component at that instant. In Fig. 14, the "active" power is shown 60 msec after the stimulus when about one third of the shortening was completed. At the right hand side of the curves is indicated the average fraction of the total power which was estimated to be derived from stored elastic energy. At the shortest length the fraction is 0.14 but at 1.10 L_b it is as large as 0.76. In view

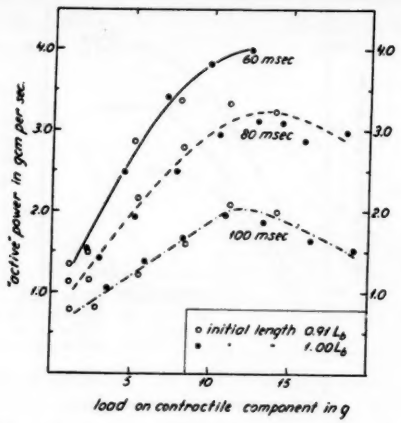

Fig. 15. The decline of the "active" power with time in locust flight muscle at 11° C (cf. Fig. 14). Lengths 0.91 L_b and 1.00 L_b.

of these large corrections, the deviations between the four curves are rather small; the curves for 0.91 L_b and 1.00 L_b actually coincide. At this instant of time (60 msec after stimulus), the length was changing rapidly. It might therefore be expected that the true dynamic force exerted by the passive system would differ considerably from the static force in the resting muscle at similar length. Fig. 14 indicates that such differences were small, even when the shortening velocity was near to its maximum at 11° C; in other words, the parallel elasticity of the flight muscle appeared to be little damped.

The change of "active" power with time is illustrated in Fig. 15 where the three curves refer to 60, 80 and 100 msec after the stimulus respectively. The "active" power became halved from 60 to 100 msec as was also the case with the total power (cf. Fig. 13). Activity had begun to decrease 60 msec after the stimulus or even earlier. During most of the shortening period, the optimum load was 10 to 15 g or 0.3 to 0.4 P_o. This corresponds to frog muscle where the power is optimal at about 0.3 P_o (BUCHTHAL and KAISER 1949; HILL 1950).

Initial shortening velocity. The shortening velocity at the beginning of the twitch is of special significance because its variation with the load on the contractile components is suited

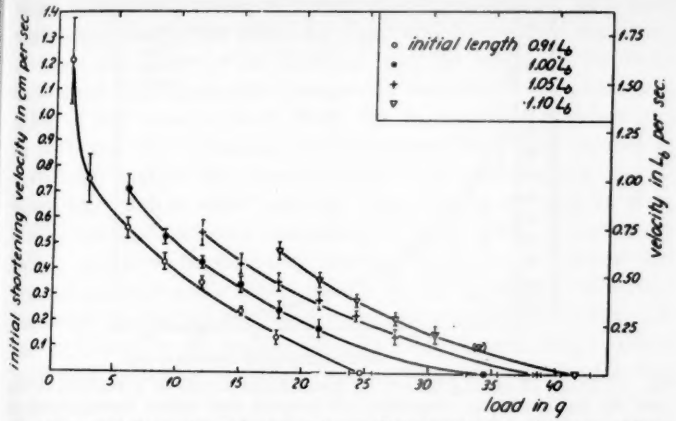

Fig. 16. Initial shortening velocity of locust flight muscle as a function of load in twitches initiated at different lengths (averaged from 7 muscles, 11° C). Vertical bars: mean errors.

for a comparison with other types of muscle according to Hill's equation (HILL 1938):

$$(P + a)\ (V + b) = (P_o + a)b.$$

P is the load, V the shortening velocity, a and b are constants, and P_o is the force developed during an isometric tetanus. The observed relationship between velocity and total force P_w is shown in Fig. 16 (11° C) but these curves could not be used directly for an estimate of a and b: (1) the resting force P_L must be subtracted; (2) the activation decreased during the twitch (the variation of P_o due to changes in length are negligible in this muscle); and (3) the isometric values in Fig. 16 correspond not to P_o but to the isometric twitch maxima which occurred 100 msec after the stimulus, *i. e.* a long time after the peak of activation. In Fig. 17, therefore, the initial shortening velocity is shown as a function of $P_w - P_L$, all values read later than 60 msec after the stimulus have been excluded, and P_o is used instead of the twitch maxima. It should now be possible to compare how the velocity depends upon the load on the contractile components at the four lengths in question. As was to be expected from the preceding analysis, the rapid but probably incomplete adjustment of the passive elastic component tended to lower the velocities of the most stretched muscles (1.05 L_b and 1.10 L_b) compared with the velocities in the muscles starting from 0.91 L_o and

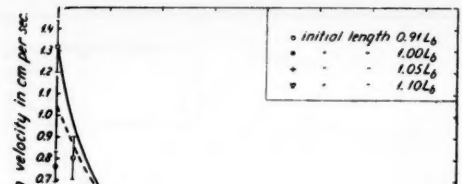

Fig. 17. Initial shortening velocity of locust flight muscle as a function of the load on the contractile component (= external load minus resting load) in twitches initiated at different lengths (averaged from 7 muscles, 11° C; cf. Fig. 16). The two curves represent theoretical force-velocity relations at length 0.91 L_b calculated from HILL's equation; full line: $a = 6.5$ g and $b = 0.25$ cm/sec; broken line: $a = 10$ g and $b = 0.32$ cm/sec; $P_o = 35$ g. Velocities taken later than 60 msec after the stimulus are omitted.

1.00 L_b. The two curves given in Fig. 17 refer to length 0.91 L_b and were *calculated* from Hill's equation using two sets of constants: $a = 350$ and 540 $g \cdot cm^{-2}$, and $b = 1.5$ and 1.8 L_b per second, respectively. The force constant a is of the same order of magnitude as in skeletal muscle of vertebrates (WILKIE 1954; ROSENFALCK and BUCHTHAL 1955); it was of the same magnitude at 20° C. As usual, the velocity constant b increased with temperature and amounted to about 6 to 9 L_b per second at 30° C. This means that in the animal, the flight muscle contracts with the same speed as human arm muscle (WILKIE 1950); the contractile system is neither faster nor stronger than skeletal muscle of mammals.

Discussion.

(a) Comparisons and General Conclusions.

The flight muscle clearly differs from other types of striated muscle but it has also many features in common with them. The tetanic force and shortening are the same as in frog muscle (WEIS-FOGH 1956 a) and the present study shows that the force constant a and the velocity constant b in Hill's equation equal

those of skeletal muscle of vertebrates. In accordance with most vertebrate muscle, the twitching flight muscle produced maximum of work and power when starting from the length in the body and when the contractile component was loaded by about one third of the tetanic force. Both the mechanical efficiency (0.1 to 0.2) and the influence of temperature find their parallel in vertebrate muscle. The general shape of the curves which characterize the "active state" and the series elastic element of HILL (1949) are dealt with in succeeding sections; both properties are rather similar to those of frog muscle. There is no indication that the actual contractile component is much specialized. However, the small shortening, the rapid metabolic recovery and, above all, the influence of the passive elasticity are features which distinguish the twitch of the flight muscle from the twitch of a frog muscle.

The small degree of shortening is not caused by incomplete activation in the beginning of the twitch (pp. 252 and 262); it must therefore be explained in terms of a relatively *quick decay* of the active state. The limited amount of shortening reduces the mechanical work done in a twitch of the flight muscle ("active" work) as compared with frog muscle because the optimum load is similar in both cases. At 20° C, the maximum twitch work is 150 to 200 gcm per cm^3 of frog muscle (calculated from BUCHTHAL, KAISER and ROSENFALCK 1951) while it was only 22 gcm per cm^3 in the flight muscle; it increased to 35 gcm at 32° C. Nevertheless, the mechanical power output of locust wing muscle is probably 4 to 8 times higher than in any vertebrate muscle hitherto studied when averaged over several motor cycles (WEIS-FOGH 1952, 1956 c). This must be due to the relatively high frequency of repetitive twitching. In flight, the twitches usually follow each other so frequently that full mechanical relaxation is just ensured (Fig. 10) and since the oxygen debt is quite insignificant as compared with the total combustion (p. 261), the metabolic recovery processes have been accelerated to such an extent that, *on average*, the recovery is complete within the brief interval of the mechanical change.

When stretched, the tension of the resting flight muscle may approach 2.5 $kg \cdot cm^{-2}$ before some fibres break; this occurs at about 1.35 L_b. The average modulus of elasticity is therefore of the same order of magnitude as in frog muscle but the relationship between load and length is about linear in the flight muscle

(BUCHTHAL and WEIS-FOGH 1956; and Fig. 4) instead of being of an exponential type as in frog muscle. In frog the resting muscle becomes more and more stiff when the length increases. This difference means that passive elastic forces are more significant in the flight muscle at its normal length in the body than they are likely to be in frog muscle. Because of the comparatively small twitch work of the flight muscle, the passive forces furthermore exert a relatively larger influence upon the total work done as well as upon the time course of the shortening than is the case in frog muscle. To understand the natural function of the flight muscle it is therefore necessary that the passive elastic force be taken into consideration. In frog muscle, it has been claimed that the resting force is due mainly or entirely to the stretching of the tough sarcolemma (RAMSEY and STREET 1940), although CASELLA's experiments (1951) strongly indicate that the content of the fibre contributes by significant amounts. In the flight muscle, similar experiments showed that the structures responsible for the major part of the passive elastic force must be located not in the sarcolemma but further inside (BUCHTHAL and WEIS-FOGH 1956).

Our experiments on the isolated flight muscle at 11° C all tend to show that the passive elastic energy can be converted into mechanical work at a rate which is of the same order of magnitude as the rate of work of the contractile system (Figs. 7B, 8, 14 and 15). At the length in the body, the "passive" part of the work at 11° C amounted to between one half (isotonic) and one third (optimum for active work) of the total twitch work.

The preceding evaluation of the passive elasticity is based mainly on indirect evidence. If it is rejected, it is necessary to postulate the existence in the flight muscle of a contractile system which differs markedly from that of ordinary striated muscle. No evidence, direct or indirect, favours this alternative. A general description of the mechanical properties of striated muscle must therefore include one term in addition to the four terms considered by HILL and recently discussed for frog muscle by WILKIE (1956). The four terms in Wilkie's presentation are (1) the active-state relationship, $P_o = f_1(t)$, (2) the stress-strain relationship of the series elastic component, $x = f_2(P)$, (3) the force-velocity relationship, $dx/dt = f_3(P)$, and (4) the tension-length relationship of the fully *activated* muscle, $P_o = f_4(x)$; t is time and x length. In the flight muscle, at least, a fifth and important term

should be added, namely (5) the relationship between length and tension of the *resting* muscle, $P_L = f_5(x)$; in return, the fourth term ($P_o = f_4(x)$), may be omitted in this muscle because of the small amount of shortening in a twitch (not in tetanic contraction).

The question arises whether the passive elastic forces are important in other types of muscle during natural activity. The mechanical advantage of an undamped or little damped spring in parallel with the contractile system is obvious in the flight muscle of locusts and other insects because it would dispose of part of the considerable negative work due to inertia of the oscillating wing (cf. MARTIN JENSEN 1956) without involving any activation or other kinds of energy expenditure. The disadvantage is that it may become a hindrance instead of a help at frequencies other than the normal stroke frequency and that it may be a nuisance if the muscle has to work at greatly different lengths. Apart from insect flight muscle, one would therefore only expect to find the passive elastic system well developed in muscles which move rather specialized, rapidly oscillating parts, for instance the wings of small birds, the tail of the rattle snake, some respiratory muscles or certain pectoral muscles of small fish. Nothing is at present known about the mechanical properties of these tissues.

(b) Active State.

According to HILL (1949) the state of activity of contracting muscle is described by means of the load P_i which the contractile component can just carry without lengthening. For frog muscle HILL found P_i to rise abruptly to a maximum value shortly after stimulation; after a "plateau of full activity" (HILL 1953), P_i declines gradually to zero. RITCHIE (1954 b) has shown that the duration of the active state increases with increasing stretch of the muscle.

By definition active state curves can be constructed from twitch maxima, since the load at the maximum gives the contraction intensity P_i at the corresponding time and length. The twitch maxima of locust flight muscle at 11° C gave the active state curves in Fig. 18 (isometric, isotonic and afterload conditions). The course of the curves is essentially similar to those given by RITCHIE (1954 b) for frog muscle but there was hardly

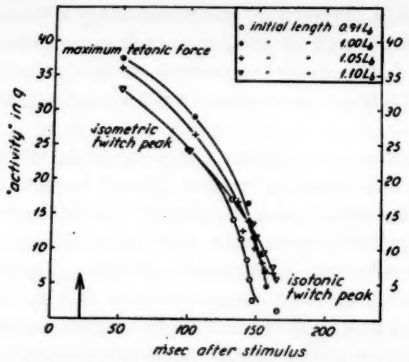

Fig. 18. "Active state" curves of locust flight muscle at four initial lengths, based upon twitch maxima at 11° C. In twitches where shortening occurred, the "activity" is the force $P_W - P'_L$ just before stretching sets in (P_W = external load; P'_L = force of resting muscle at the shortest length). The arrow indicates the onset of the mechanical change which ends 320 msec later. It is assumed that the "activity" at the onset of shortening equals the maximum tetanic force.

any time for a "plateau" nor any significant differences in the duration of the active state at the four lengths considered here. The activation of the contractile substance seems to be of the same nature as in other striated muscles and to be nearly independent of the type of contraction. The maximum rate of decline was 360 g per second at 11° C, corresponding to about 11 P_o per second. It is about 5.5 P_o per second in frog muscle at 0° C (RITCHIE 1954 b).

(c) Series Elastic Element.

The mechanical properties of active and resting muscle are by HILL (1949) interpreted by means of a three-element model, viz.: (1) a contractile component obeying a characteristic relation between load and shortening velocity, (2) an elastic component in series with the contractile one, and (3) an elastic component in parallel with the other components.

The load-extension diagram of the series elastic component may be determined from the isometric twitch curve provided the relation between load and shortening velocity of the contractile component is known (HILL 1949). For locust flight muscle this indirect procedure resulted in the relations shown in Fig. 19 (11° C) where the load given is the total load carried by the muscle. The curves refer to times later than 30 msec after the

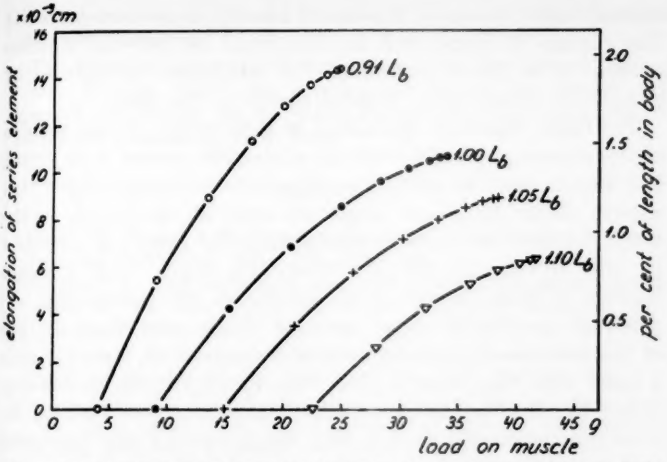

Fig. 19. Load-extension diagrams of the series elastic element of locust flight muscle at different initial lengths. The curves were calculated from the isometric twitch curves (Fig. 11) and the force-velocity relations (Fig. 13). They refer to times later than 30 msec after the stimulus (11° C).

stimulus, *i. e.* 10 msec after the end of the mechanical latency, the shortening velocity being unknown before this time. The method is not very accurate because any type of slack in the recording system will appear as a series compliance. In toad muscle HILL (1949) found an extensibility of the series element corresponding to 1 per cent of the muscle length per 0.2 to 0.3 kg · cm^{-2} and in single frog fibres BUCHTHAL, KAISER and ROSENFALCK (1951) found 1 per cent per 0.4 kg · cm^{-2}. In the locust muscle the figure was about 1 per cent per 0.8 kg · cm^{-2}, *i. e.* the series elastic element estimated by this method is about twice as stiff in the flight muscle. This would explain why the isometric force increased twice as fast in the flight muscle as in frog muscle. A more direct investigation of the series element by quick stretch and quick release experiments is desirable, as are also dynamic experiments on the parallel elastic component of resting flight muscle.

Summary.

A new type of myograph (Fig. 1) was used to study the mechanical changes during twitch contractions of the striated, longi-

tudinal flight muscle of the desert locust (*Schistocerca gregaria*). The changes in length and in force could be recorded simultaneously or in quick succession. The results are interpreted by comparison mainly with striated muscle of the frog.

The flight muscle is specialized in three ways: (1) the passive elastic system in parallel with the contractile system is so strong and adjusts itself so quickly to alterations in muscle length that passive elastic forces are important even at the length in the body and may become dominating when the muscle is stretched by only five or ten per cent above this length. Only a minor fraction of these passive forces are due to the sarcolemma. (2) Although the "active state" curve is of the same shape as that of the frog muscle, the duration of the activation lasts for such a short time that there is little time for shortening to develop. When the load on the contractile component was optimal for work production (0.3 P_c; Fig. 7B), the shortening only amounted to 3 or 4 per cent of the muscle length (11° C; Fig. 6), as compared with about 25 per cent in frog muscle at 0° C. (3) In the flying locust the wing stroke frequency (= twitch frequency) is equal to or little less than the frequency at which mechanical summation begins at the thoracic temperature for flight (30° to 42° C); since the oxygen debt is insignificant, the metabolic recovery must be complete by the end of the mechanical change. It may be added that contrary to frog muscle, the twitch force of flight muscle increased with rising temperature.

If parallel elastic forces are allowed for, the mechanical properties of the contractile or "active" components are similar to those in skeletal muscle of vertebrates. This applies both to the force parameter a in HILL's equation and to the velocity parameter b as well as to the relationship between "active" work and load. The shortening velocity reached a maximum at the beginning of the shortening; in contrast to findings in frog muscle it declined linearly with time from then on so that no phase of constant velocity exists. The series elastic element appeared to be of the same nature as in frog muscle but may be twice as stiff.

The existence of a strong, passive elasticity in parallel with the contractile system, even at the length in the body, must be taken into consideration for an understanding of the mechanics of flight muscle in addition to the active state curve, the series elastic element, and the force-velocity relation.

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Studies on the Placental Transfer of Phosphate in the Guinea Pig.

III. Phosphorus Compounds in the Placenta and their Uptake of Radioactive Phosphorus.[1]

By

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Received 15 February 1957.

In a previous paper we have described experiments on the transfer of inorganic phosphate from mother to foetus in the guinea pig, using radioactive phosphorus as a tracer (FUCHS and FUCHS 1957). It was pointed out that in experiments of relatively short duration not only the foetal uptake of radioactive phosphorus but also the placental uptake had to be taken into consideration. The foetuses and placentas were delivered in most cases half an hour after the injection of radiophosphate into the mother, because within this interval the return of labelled phosphate from foetus to mother could be regarded as negligible. However, not all the phosphate taken up by the placenta from the maternal plasma can be regarded as phosphate "in transfer" to the foetus. Small quantities are retained in placental growth, and besides there might be an exchange of phosphate between the maternal plasma and the placental cells not related to the supply of phosphate to the foetus.

In order to obtain more detailed information on the placental uptake of phosphate, we determined the content of inorganic

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phosphate and various organic phosphorus compounds and measured the uptake of radioactive phosphorus in these fractions in thirteen of the previously described experiments (FUCHS and FUCHS 1957), carried out between the 29th day of gestation and term. In one additional case the phosphorus compounds were determined without radiochemical measurements. The following fractions were determined: total phosphorus; inorganic phosphate; organic acid-soluble phosphorus and the following fractions thereof: phosphocreatine, adenosine di- and triphosphate, and hexose monophosphates. The organic acid-insoluble fraction, including phospholipids, phosphoproteins and nucleic acids, was taken as the difference between the total phosphorus and the total acid-soluble phosphorus (inorganic phosphate plus organic acid-soluble phosphorus).

Procedure.

The placentas to be analyzed were frozen in a mixture of solid carbon dioxide and alcohol immediately after delivery without separation of the subplacentas. A part of the frozen specimen was chiselled off, weighed, and ashed for total phosphorus determination as previously described (FUCHS and FUCHS 1957). The remaining part was rapidly weighed and then homogenized in an Elvehjem homogenizer with four volumes of chilled 10 % trichloracetic acid, and extracted for 20 minutes. The samples were then centrifuged in tubes immersed in crushed ice. The precipitates were washed twice with ice-cold 5 % trichloracetic acid. The supernatants from each sample were added and diluted, and kept at 0° C until neutralized.

The trichloracetic extracts were fractionated as described by SACKS (1949) with slight modifications. The amount of radioactive phosphorus in each fraction was determined after precipitation of the phosphorus as magnesium ammonium phosphate. Inactive phosphate was added to samples of low phosphorus content to give precipitates of equal weight (LEVI 1941).

Results.

The results are shown in Table 1. To facilitate comparison of the principal fractions these have been shown on Fig. 1, which includes data from the experiment where no radiochemical determinations were made (Exp. No. 2). It is seen that the placental content of total phosphorus is fairly constant throughout the latter half of gestation, varying between 1.56 and 2.34 mg with an average of 2.0 mg phosphorus per gram wet weight. The total acid-soluble phosphorus increases slightly until about the 60th

Table 1.

The phosphorus compounds in the placenta and their specific activities relative to that of orthophosphate half an hour after intravenous injection of ^{32}P into the mother. In Exp. No. 1 determinations were carried out on two placentas. The specific activities of the inorganic phosphate in the maternal and foetal plasma relative to that of the placental orthophosphate are shown for comparison.

Experiment No.		5	6	11	13	3	10	8	9	4	14	12	15	1[1]	1[2]
Gestation age (days)		29	32	33	39	41	43	45	46	48	52	56	57	59	59
Total phosphorus	μg P/g placenta	2,018	2,020	2,003	1,560	2,287	1,950	2,335	1,820	2,213	2,081	2,163	2,030		1,933
	rel. spec. act.	24	21	23	23	21	25	21	27	19	27	25	29		60
Total acid-soluble phosphorus	μg P/g placenta	739	773	874	763	822	1,124	849	873	792	853	1,009	945	1,088	1,096
	rel. spec. act.	53	56	49	46	51	41	53	60	50	60	58	56	70	85
Ortho-phosphate	μg P/g placenta	236	238	280	232	246	301	224	340	265	296	338	332	477	550
	rel. spec. act.	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Phospho-creatine	μg P/g placenta	13	0	3	0	7	3	35	39	0	27	0	38	0	0
Organic acid-soluble phosphorus	μg P/g placenta	503	535	594	531	576	823	590	493	527	543	671	575	611	546
	rel. spec. act.	31	35	25	22	31	18	35	43	27	7	37	34	46	68
ATP-labile-phosphorus	μg P/g placenta		74	35	43	116	32	19	24	48	28	43	64	57	52
	rel. spec. act.		122		89	69	97	78	149	103	138	48	53	224	465
Glucose-1-phosphate	μg P/g placenta	17	23	18	32			17	44		20	32	29	8	21
	rel. spec. act.	71	88	85	66			116	50				86	108	58
Glucose-6-phosphate	μg P/g placenta	204	130	59	208			51	116				71	330	110
	rel. spec. act.	1	10		5			36	9				28	3	2
Total acid-insoluble phosphorus	μg P/g placenta	1,279	1,247	1,129	797	1,465	826	1,486	947	1,421	1,228	1,022	1,085		737
	rel. spec. act.	7		4	1	4	5	3	0	1	0	0	6		24
Maternal plasma inorganic phosphate	μg P/g plasma	67	51	41	46	40	45	46	45	42	37	40	43	43	43
	rel. spec. act.	372	350		548	300	264	276	322	242	258	231	257	359	359
Foetal plasma inorganic phosphate	μg P/ml plasma							90		94	87		70		
	rel. spec. act.							56		45	76		52		

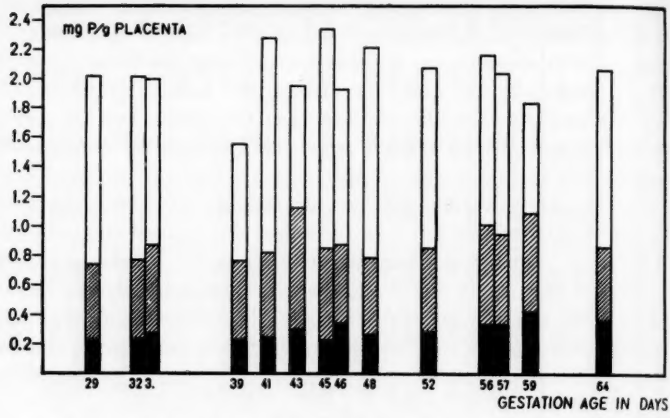

Fig. 1. Principal phosphorus compounds in the guinea pig placenta at various stages of gestation. Solid columns = orthophosphate; hatched columns = organic acid-soluble phosphorus; open columns = acid-insoluble phosphorus. The whole columns indicate total phosphorus.

day, after which it seems to decrease. Unfortunately, only one determination has been made in the last week of gestation. The increase in the acid-soluble phosphorus must be larger in the placental cells than in the placenta as a whole, on account of the increasing amount of maternal and foetal blood which contains relatively little acid-soluble phosphorus (FUCHS 1953). The increase is mainly in the inorganic phosphate, but also the organic acid-soluble fraction increases slightly toward the 60th day.

Phosphocreatine could not be demonstrated in all instances, but in some cases small amounts were found. The glucose-1-phosphate concentration is low, varying between 0.8 and 4.4 mg per cent, while glucose-6-phosphate is present in a somewhat higher concentration, with an average of 14.0 mg per cent (5.1—33.0 mg per cent). The concentration of ADP- and ATP-labile-phosphorus varies between 1.9 and 7.4 with an average of 4.3 mg per cent.

Table 1 also shows the specific activity of the various fractions as a percentage of the specific activity of the orthophosphate fraction (specific activity defined as the radioactivity per mg phosphorus). The specific activity of the inorganic phosphate in the maternal and the foetal plasma relative to that of the placental orthophosphate has also been indicated. In all the experiments

Table 2.

The content of various phosphorus compounds in the guinea pig placenta in the middle and last part of gestation, and in the uterine muscle during gestation in the rat (WALAAS *and* WALAAS *1950*), *the rabbit* (MENKES *and* CSAPO *1952*), *and the human* (KINNUNEN *and* PEKKARINEN *1952*), *expressed in μg P/g wet weight.*

	Guinea pig placenta		Uterine muscle in		
	29—32 d.	56—59 d.	rat	rabbit	human
Total phosphorus	2,010	2,010	—	—	1,050
Total acid-soluble phosphorus .	795	978	484	—	560
Orthophosphate phosphorus ...	251	421	183	72	290
ATP-labile-phosphorus	54	55	34	62	60
Phosphocreatine phosphorus ..	3	13	14	55	—
Organic acid-sol. phosphorus ..	544	600	301	—	270
Acid-insoluble phosphorus	1,210	950	—	—	490
Lipid phosphorus	—	—	—	—	480

except Nos. 13 and 15, the interval between injection of radioactive phosphate into the mother and delivery of the placenta was about thirty minutes; in Exp. No. 13 it was seventeen minutes and in No. 15 it was fifty minutes. It is seen that after half an hour the placental orthophosphate has obtained about one third of the specific activity of the inorganic phosphate in the maternal plasma.

Nearly all the radioactive phosphorus taken up by the placenta is found in the acid-soluble fraction. The organic acid-soluble fraction has a specific activity which is about one third of that of the orthophosphate.

The ADP- and ATP-labile-phosphorus fraction which also contains some hexose diphosphate phosphorus is apparently completely in equilibrium with the orthophosphate. Excluding the extremely high value from experiment No. 1, the average relative specific activity is 99. Glucose-1-phosphate also has a high relative specific activity (average 84), while that of the glucose-6-phosphate is low (average 15).

The relative specific activity of the inorganic phosphate in the foetal plasma is 50—60 after half an hour. This is about one sixth of the relative specific activity of the maternal plasma phosphate.

Discussion.

The evaluation of the relative quantities of the various phosphorus fractions is complicated by the large amounts of blood in the placenta. Lacking determinations of the phosphorus fractions of the maternal and foetal blood, and knowing the blood volume of the foetal vessels of the placenta only in the last part of gestation (FUCHS 1953), it is not possible to estimate the fractions within the placental cells. This of course complicates fractionation in every type of animal tissue, but in few tissues is the blood volume as large as in the placenta. This should be borne in mind when values for the placenta and the uterine muscle are compared, as has been done in Table 2.

It is of special interest to note the high concentration of inorganic phosphate in the placenta. Since the blood contains but little inorganic phosphate, the concentration within the placental cells must be even higher than in the organ as a whole. With a specific activity between that of the inorganic phosphate in the maternal plasma and that of the foetal plasma phosphate, it appears to form a reservoir, from which the foetal plasma could acquire phosphate by diffusion. To build up the high concentration in the placental tissue from the low concentration in the maternal plasma would, on the other hand, seem to require an active transport mechanism. This is further discussed elsewhere (FUCHS 1957).

Summary.

Determinations of the various phosphorus fractions in the guinea pig placenta in a series of experiments on the placental transfer of phosphate have shown that the orthophosphate concentration is very high in the placental tissue as compared with the maternal and foetal plasma. The concentration increases slowly from the 29th to the 59th day of gestation and may decrease thereafter.

Thirty minutes after intravenous injection of radioactive phosphate into the mother the orthophosphate in the placenta has reached a specific activity of one third of that of the inorganic phosphate in the maternal plasma. It has the highest specific activity of the placental phosphorus fractions.

Adenosine diphosphate and triphosphate are found in approximately the same concentrations as in the uterine muscle during pregnancy in the rat, the rabbit and the human. The labile phosphorus of these compounds is in equilibrium with the orthophosphate with regard to radioactive phosphorus.

Glucose-1-phosphate is present in a low concentration, but it acquires a high relative specific activity. More glucose-6-phosphate is present, but with a low relative specific activity.

The inorganic phosphate in the foetal plasma obtains in thirty minutes a specific activity of about half that of the placental orthophosphate.

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The Fibrinolytic Activity of Animal Tissues.

By

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ASTRUP and PERMIN (1947) demonstrated an activator of plasminogen in animal tissues. Though this observation has been repeatedly confirmed the insolubility of the activator has prevented its quantitative estimation. ASTRUP (1952) demonstrated also a potent protease inhibitor (pulmin) in ox lung tissue. This inhibitor was absent from the lungs of a number of other species including calf embryo.

Later ASTRUP and STAGE (1952) extracted the activator from pig hearts with potassium thiocyanate. Based on this observation a method was devised for the simultaneous quantitative determination of the tissue activator and the inhibitor (ASTRUP and ALBRECHTSEN 1957). The present paper reports the concentrations of the activator and the inhibitor in tissues from various animals, including the calf embryo.

Materials and Methods.

Samples of tissue were removed immediately after killing and investigated on the same day or left for later use at — 20° C. Only organs from apparently healthy animals were used.

The tissue activator concentration and the content of the inhibitor was estimated according to ASTRUP and ALBRECHTSEN (1957). Activator activity was estimated on standard fibrin plates (ASTRUP and MÜLLERTZ 1952) and inhibitor activity against trypsin on heated fibrin plates (LASSEN 1952). The concentration of the tissue activator is expressed in units of a standard preparation per gram of fresh tissue.

The stock solution of trypsin (Novo "Trypure": 24.4 Anson units per gram) contained 0.25 mg per ml in 0.025-N HCl and was diluted before use 2 to 15 in barbital buffer (dietyl barbiturate: 0.05-M; pH 7.8; containing 0.1-M NaCl. Total μ: 0.15).

Results and Discussion.

The concentration of the tissue activator varies in the same organ from species to species and within the same species from animal to animal (Table 1). The brain, ovary, uterus, adrenal gland and the prostate of most of the animals were active. Lung tissue from most of the animals (especially the mouse and rat) was active; the pig, ox and rabbit lung was inactive. This result is not in accordance with investigations by ASTRUP (1952) and ASTRUP and STERNDORFF (1956) who found pig lung to be a potent activator of plasminogen. However, occasionally an activity was found in samples from pig lung. These discrepancies are at present unexplained. The pig heart was a good source of the tissue activator. More moderate activity was found in the heart from the horse, ox, guinea-pig and rat. Heart tissue from the dog, rabbit, cat and mouse was inactive. Only in two species activity was found in the sceletal muscles (the pig and horse). The testis contained moderate activity in most animals, but the pig, rabbit and cat were inactive. A moderate activity (not included in the table) was recorded in the kidney from the horse, and a trace of activity in kidneys from the mouse, rat and guinea-pig. All other kidney samples were inactive. Only one sample from the spleen (guinea-pig) was active, all the other samples and all samples from the liver were inactive. Tissues from cod-fish and fowl were completely inactive.

This study confirms the earlier observations that fibrinolytic activity in animal tissue is caused by an activator of plasminogen (ASTRUP and PERMIN 1947, PERMIN 1950, TAGNON and PETERMANN 1949, LEWIS and FERGUSON 1950, ASTRUP and STERNDORFF 1952.) The distinction between fibrinolytic enzymes and activators of plasminogen was made possible by the use of standard fibrin plates (containing plasminogen) and of heated fibrin plates (containing no plasminogen) (LASSEN 1952). The tissue extracts digested the substrate in the standard fibrin plates whereas no digestion was obtained on the heated fibrin.

PERMIN (1950) showed that the fibrinolytic activity of some calf embryo organs was higher than in the adult cow. The activities of different organs from 14 calf embryos (of varying age) and from 5 calves were therefore estimated (Table 2). The concentration in the heart is almost unchanged during foetal life and

Table

Plasminogen activator concentration of different animal tissues. Activities The three values refer to three different samples. + represents

Organ	Pig			Horse			Ox			Dog		
Brain	69	32	32	130	69	22	75	75	54	11	11	5
Lung	0	0	0	97	45	43	0	0	0	23	12	8
Heart	216	216	216	45	25	25	34	17	34	0	0	0
Uterus	313	76	47	180	126	108	9	0	0	10	58	54
Muscles ...	50	22	0	108	108	38	0	0	0	0	0	0
Prostate ..	5	5		238	65	180	0	0	11	216	108	76
Adrenal...	97	37	32	30	17	8	65	34	15	37	37	24
Testis	0	0		32			11	11	14	19	11	6
Ovary	292	130	97	36	25	22	23	8	6	25	54	8

similar to the concentration in calf and ox. Quite differently the activator concentration in lung is moderate until late foetal life when it disappears. Very small amounts of activator were observed in two only of five samples from the calf lung and none was present in the ox lung. In kidney the tissue activator concentration was moderate in the embryo and the calf but absent in the ox. Additional results appear from Table 2.

Inhibitory compounds were found only in bovine lung and uterus (Fig. 1). One sample from the bovine uterus contained inhibitory compounds as well as small amounts of the activator. Small amounts of inhibitory compounds were found also in the lungs of two late calf embryos (Table 2). Larger amounts were found in calf lung and even larger amounts in the ox lung (Fig. 2). No inhibitory effect against trypsin was recorded in any of the other tissues from the calf embryo or from the calf.

The concentration of tissue activator in human tissues is on an average much larger than the concentration in animal tissues (ALBRECHTSEN 1957). However, most of the active animal tissue (lung, uterus, prostate, adrenal gland and ovary) are the same as the most active human tissues.

1.

expressed in units of a standard preparation per gram of fresh tissue. samples which produced only a slight digestion of the substrate.

Rabbit			Guinea-pig			Cat			Mouse			Rat		
+	+	0	25	29	18	0	0	0	13	+	+	19	27	29
+	0	0	14	18	12	25	16	0	238	216	184	248	245	203
0	0	0	32	50	36	0	0	0	0	0	0	19	19	23
63	40	40	68	32	36	14	27	24	+	+	7	14	12	5
0	0	0	0	0	0	0	0	0	0	0	0	0	+	0
0	0	0												
0	0	0	8	5	6	36	17	7				77	58	45
0	0	0	31	9	5	0	0	0						
30	27	8				+	10	7				99	54	29

The activator and the inhibitory compounds behave differently in the pre- and postnatal state: in calf embryo inhibitory compounds were absent while they were present in the lung of calf and ox. Activator was absent in lung, kidney, spleen, liver and muscle from adult cow but was present in the calf embryo. Both compounds were very rarely present in the same tissues.

Summary.

1. The concentration of the tissue activator of plasminogen was determined in organs from various animals and from the calf embryo.

2. In general brain, lung, heart, uterus, prostate, adrenal and ovary contained moderate to large amounts of activator. With few exceptions kidney, spleen, muscles and liver did not contain activator.

3. Protease inhibitors (against trypsin) were found only in bovine lung and uterus.

4. Calf embryo tissues contained more activator than tissues from the adult cow.

Table 2.

Activity of different tissues from the calf embryo, the calf and the adult animal. Activities expressed as in Table 1. +i. represents samples containing inhibitory compounds.

Length	Heart	Lung	Kidney	Spleen	Liver	Muscles	Brain	Adrenal	Testis
7 cm		32			0		19		
9 cm	47	17			0		20		
20 cm	68	32	34	0	45	0	0		
30 cm	81	9	14	0	14	0	7		
36 cm	61	14	17	14	0	0	0		
50 cm	68	19	23	19	3	3	7	68	
58 cm	79	45	30	13	18	6	18	146	
65 cm	27	19	14	9	0			28	8
70 cm	34	23	23	17	5	8			
85 cm	39	14	39	7	1	2		72	
90 cm	42	27	10	7	0		6		16
132 cm	72	9 +i.	34	9	0	0	26	63	
145 cm	5	5	7	3	0	0	5	23	
155 cm	42	0 +i.	28	12	0	0	34	34	
Calf	28	14 +i.	20	7	0				14
Calf		6 +i.							
Calf	39	0 +i.	12	3	0	0		30	0
Calf		0 +i.							
Calf		0 +i.							
Adult ox ..	17	0 +i.	0	0	0	0	75	65	11
Adult ox ..	34	0 +i.	0	0	0	0	75	34	11
Adult ox ..	34	0 +i.	0	0	0	0	54	15	14

5. The trypsin inhibitor appeared in the embryo lung during late foetal life.

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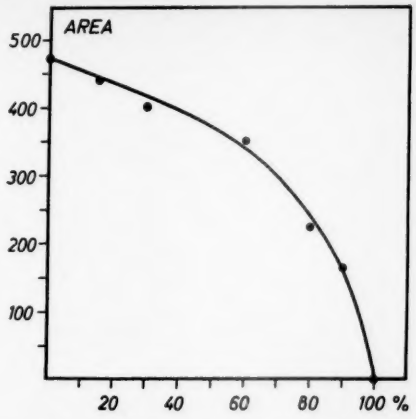

Fig. 1. Inhibition of trypsin by extracts from the bovine uterus. 0.5 ml of the different dilutions of the tissue extract prepared as described by ASTRUP and ALBRECHTSEN (1957) was added to 0.5 ml of the trypsin solution and the remaining trypsin activity estimated on heated fibrin.
Abscissa: Concentration of tissue extracts in % of the stock solution. *Ordinate:* Trypsin activity as product of two diameters (mm^2) of the lysed zones.

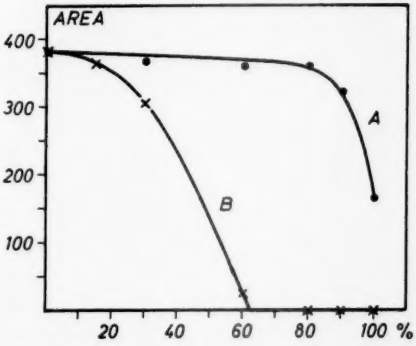

Fig. 2. Inhibition of trypsin by tissue extracts from bovine lung. Embryo (A). Adult animal (B). Experiments performed as in fig. 1. Abscissa and ordinate as in fig. 1.

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